β-Arrestin-Biased Agonists of the GLP-1 Receptor from β-Amino Acid Residue Incorporation into GLP-1 Analogues

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Ⓢ *Supporting Information*

ABSTRACT: Activation of a G protein-coupled receptor (GPCR) causes recruitment of multiple intracellular proteins, each of which can activate distinct signaling pathways. This complexity has engendered interest in agonists that preferentially stimulate subsets among the natural signaling pathways ("biased agonists"). We have examined analogues of glucagon-like peptide-1 (GLP-1) containing β-amino acid residues in place of native α residues at selected sites and found that some analogues differ from GLP-1 in terms of their relative abilities to promote G protein activation (as monitored via cAMP production) versus β-arrestin recruitment (as monitored via BRET assays). The α → β replacements generally cause modest declines in stimulation of cAMP production and β-arrestin recruitment, but for some replacement sets cAMP production is more strongly affected than is β-arrestin recruitment. The central portion of GLP-1 appears to be critical for achieving bias toward β-arrestin recruitment. These results suggest that backbone modification via α → β residue replacement may be a versatile source of agonists with biased GLP-1R activation profiles.
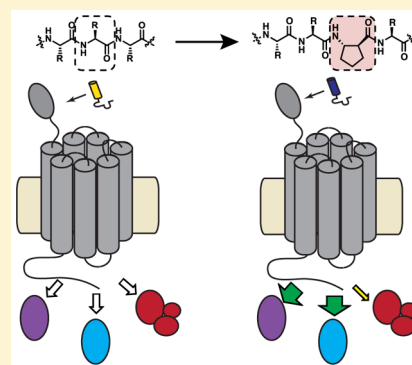
INTRODUCTION

G protein-coupled receptors (GPCRs) play critical physiological roles by transmitting information encoded in extracellular molecules to the cytoplasm. Signal transduction requires interaction of an activated GPCR with intracellular partner proteins, which can include several G proteins ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, etc.), β-arrestin-1, and β-arrestin-2 (Figure 1A). Each of these effector proteins stimulates one or more signaling pathways. $G\alpha_s$, for example, leads to the production of the second messenger cAMP, $G\alpha_q$ leads to mobilization of intracellular Ca^{2+}, and β-arrestins mediate receptor internalization and/or serve as scaffolds for G protein-independent signaling.[1−3] GPCR structure is dynamic, and each effector protein is thought to bind to a unique receptor conformation.[4,5] GPCR ligands that favor engagement of certain effector proteins over others, referred to as "conformationally selective" or "biased" agonists, are believed to favor a subset among the activated GPCR conformations (a hypothetical, idealized example of a β-arrestin-biased agonist is shown in Figure 1B; in contrast, the bias we report here stems from diminished activity in all channels relative to the native agonist, with greater diminution in some channels relative to others).[6−8] Mechanistic understanding of ligand bias remains limited, despite extensive study.[9−11] Little structural information is available for GPCR complexes of biased agonists,[12,13] which hinders both rational design of agonists with pathway selectivity and molecular-level elucidation of the origins of bias.

The glucagon-like peptide-1 receptor (GLP-1R) is a member of the B subclass of GPCRs, which are activated by long polypeptide hormones. GLP-1R is primarily expressed in pancreatic β cells, which secrete insulin to regulate glucose concentration in the bloodstream. This receptor can be engaged by multiple endogenous peptides, including at least six forms of glucagon-like peptide-1. The full-length forms, GLP-1(1−36)NH₂ and GLP-1(1−37), are considered weak agonists or antagonists of GLP-1R.[14] The mature forms, GLP-1(7−36)NH₂ and GLP-1(7−37), are potent agonists; these two forms display indistinguishable activity.[14,15] After release into the bloodstream, the mature forms of GLP-1 are rapidly cleaved by dipeptidyl peptidase-4 (DPP-4), to generate GLP-1(9−36)NH₂ and GLP-1(9−37), which are low-affinity, low-efficacy agonists of GLP-1R-mediated signaling and can function as antagonists.[14] In addition, the peptide hormones glucagon[16] and oxyntomodulin[16,17] (which is an eight-residue extension of glucagon) can activate GLP-1R (sequences shown in Figure 2).

Binding of the mature forms of GLP-1 (referred to below simply as "GLP-1") to the GLP-1R is associated with diverse effects that are critical for human health, but the intracellular signaling networks initiated in this way have not yet been fully elucidated. GLP-1 binding to the GLP-1R activates $G\alpha_s$ and
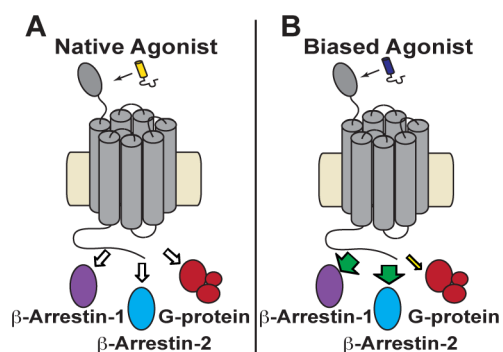
Figure 1. Schematic depicting the intracellular proteins, investigated in the current study, that are recruited to a GPCR upon stimulation with (A) the native receptor agonist or with (B) a β-arrestin-biased agonist. In (A), equally sized and colored arrows indicate equal recruitment of each potential partner protein upon stimulation with the native agonist, while in (B) larger green arrows pointing to β−arrestin-1 and β−arrestin-2 indicate a greater level of β-arrestin recruitment upon stimulation with a β-arrestin-biased agonist relative to the native agonist, and the smaller yellow arrow pointing to a G protein indicates a diminished level of G protein recruitment upon stimulation with a β-arrestin-biased agonist relative to the native agonist.

thereby induces a rise in intracellular cAMP, which leads to glucose-stimulated insulin secretion.[18] GLP-1 binding recruits also β-arrestin-1 and β-arrestin-2 to the GLP-1R. Recruitment of β-arrestin-1 promotes β cell proliferation[19] and protects these cells from apoptosis.[20,21] The effects of GLP-1-induced β-arrestin-2 recruitment to the GLP-1R are not yet clear.[22] GLP-1R ligands that manifest strong selectivity in terms of effector protein recruitment and activation would be valuable tools for elucidating the role(s) of individual effector proteins.

Synthetic peptide agonists of GLP-1R have been developed to treat type II diabetes by enhancing glucose-stimulated insulin secretion from pancreatic β cells, among other mechanisms. Exendin-4, for example, is a component of the venom of the Gila monster and functions as a potent agonist of this receptor.[23] Liraglutide is nearly identical in sequence to GLP-1(7−37) but bears a hexadecanoyl appendage on the side chain of Lys-26.[24] This appendage promotes binding to serum albumin, which protects liraglutide from DPP-4 cleavage and dramatically enhances lifetime in the bloodstream.[25]

Variations in the α-amino acid sequences of GLP-1R agonists lead to differences in the relative and absolute abilities of these agonists to induce recruitment of intracellular effector proteins to the receptor and differences in the stimulation of downstream signaling events. Thus, for example, relative to mature GLP-1, both exendin-4 and oxyntomodulin show a bias toward recruitment of β-arrestin-1 and β-arrestin-2 relative to cAMP generation (which presumably reflects Gα_s activation).[26]

Zhang et al. recently reported discovery of an exendin-4 derivative that displays significant bias in favor of G protein activation, which leads to an altered in vivo efficacy profile relative to exendin-4.[27] Furthermore, allosteric GLP-1R ligands can influence the pattern of intracellular protein recruitment induced by a given peptide agonist.[28,29] These observations are of great interest from the biomedical perspective, because it has been proposed that therapeutic benefits may be maximized (and deleterious side effects minimized) with drugs that exert specifically tailored activating effects on the GLP-1R (or other GPCRs).[6,30,31] However, the origins of ligand-dependent variations in signaling outcome are not yet clear at the molecular level, and the rational design of agonists with predetermined bias profiles at a given GPCR is currently not possible.

Here we show that GLP-1 analogues containing β-amino acid residues can display substantial bias in terms of intracellular partner engagement by the GLP-1R. These findings are significant because nearly all known GLP-1 analogues are composed exclusively of α-amino acid residues, although a few examples with unnatural backbone components have been described.[32−34] β residue incorporation is simple in the context of conventional solid-phase peptide synthesis, and the findings disclosed here suggest that evaluation of small sets of peptide hormone analogues containing α → β replacements could be a generally productive strategy for discovery of molecules with mechanistic and perhaps ultimately therapeutic utility.

■ RESULTS

Peptide Design. We previously described a small set of GLP-1(7−36)-NH2 analogues, including **1−4** (Figure 3), that contain α → β replacements in the C-terminal region of the hormone and retain agonist activity at the GLP-1R.[35] The most heavily modified member of this set, **4**, contains two α → Aib (2-aminoisobutyric acid) substitutions as well as five α → β substitutions. The β residue placement in this set conforms to an ααββ pattern. The ring constraint in the β residues employed in **1−4** is known to support adoption of an α-helix-like conformation by α/β-peptides;[36] a cocrystal structure of GLP-1 and the GLP-1R extracellular domain shows that the C-terminal portion of the hormone adopts an α-helical conformation in the bound state.[37]

In the previous study we sought to maximize β residue content in order to minimize the susceptibility of our α/β-peptides to proteolytic degradation. Extension of the ααββ pattern evident in **3** to a sixth site (position 14), however, caused a substantial decline in agonist activity.[35] We therefore turned to α → Aib replacements in the N-terminal portion to enhance protease resistance. GLP-1 is rapidly cleaved between Ala8 and Glu9 by dipeptidyl peptidase-4 (DPP-4), but this

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                        1    5    10   15   20   25   30   35
GLP-1 (1-37)       HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-OH
GLP-1 (1-36)NH2    HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2
GLP-1 (7-37)            HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG-OH
GLP-1 (7-36)NH2        HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2
GLP-1 (9-37)              EGTFTSDVSSYLEGQAAKEFIAWLVKGRG-OH
GLP-1 (9-36)NH2          EGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2
Glucagon               HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH
Oxyntomodulin          HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNKNNIA-OH
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Figure 2. Amino acid sequences of each endogenous form of GLP-1, as well as the sequences of related peptides glucagon and oxyntomodulin.
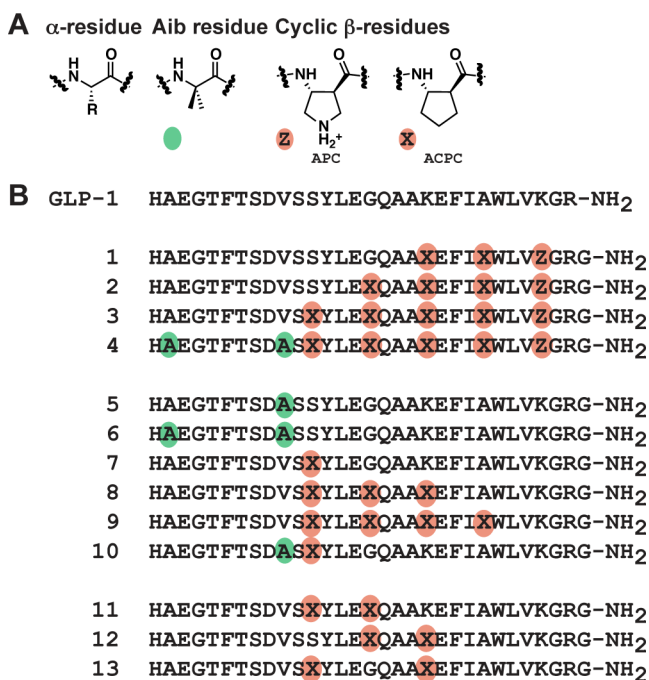
A α-residue Aib residue Cyclic β-residues

B GLP-1 HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH2

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 1  HAEGTFTSDVSSYLEGQAAXEFIXWLVZGRG-NH2
 2  HAEGTFTSDVSSYLEXQAAXEFIXWLVZGRG-NH2
 3  HAEGTFTSDVSXYLEXQAAXEFIXWLVZGRG-NH2
 4  HAEGTFTSDASXYLEXQAAXEFIXWLVZGRG-NH2

 5  HAEGTFTSDASSYLEGQAAKEFIAWLVKGRG-NH2
 6  HAEGTFTSDASSYLEGQAAKEFIAWLVKGRG-NH2
 7  HAEGTFTSDVSXYLEGQAAKEFIAWLVKGRG-NH2
 8  HAEGTFTSDVSXYLEXQAAXEFIAWLVKGRG-NH2
 9  HAEGTFTSDVSXYLEXQAAXEFIXWLVKGRG-NH2
10  HAEGTFTSDASXYLEGQAAKEFIAWLVKGRG-NH2

11  HAEGTFTSDVSXYLEXQAAKEFIAWLVKGRG-NH2
12  HAEGTFTSDVSSYLEXQAAXEFIAWLVKGRG-NH2
13  HAEGTFTSDVSXYLEGQAAXEFIAWLVKGRG-NH2
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Figure 3. (A) α-Amino acid and β-amino acid residues. The colored circles used to indicate non-natural substitutions in sequences below are defined: green circles represent the α residue Aib, and orange circles represent ring-constrained β residues (X = ACPC, Z = APC). (B) GLP-1(7−36)NH2 and α/β-peptide analogues **1−13** (based on GLP-1(7−37)NH2). Each peptide has a free N-terminus and a primary amide at the C-terminus.

cleavage can be blocked with maintenance of agonist activity by replacing Ala8 with Aib.[38] Aib was therefore placed at position 8 in α/β-peptide **4**. The other protease known to degrade GLP-1 in vivo is neprilysin,[39] which cleaves at many sites in the C-terminal and central regions of the hormone. We introduced Aib at position 16 in **4** to augment the protection from neprilysin expected from the five α → β substitutions in **3**.[35]

Our initial studies of GLP-1R activation by α/β-peptides[35] focused on cAMP accumulation; the work described here expands this evaluation to include recruitment of β-arrestin-1 and β-arrestin-2, as well as a more sensitive alternative assay for cAMP production. In an effort to elucidate the origin of pathway selectivities detected among α/β-peptides **1−4**, we expanded this collection to include **5−13** (Figure 3), analogues of GLP-1(7−37)NH2 that contain different subsets among the α → β and/or α → Aib replacements found in **4**. Although GLP-1(7−37)NH2 does not occur naturally, this peptide is as

potent as the two mature forms of the hormone.[40] One of the natural mature forms, GLP-1(7−36)NH2, serves as the reference agonist in these studies; we found that GLP-1(7−36)NH2 and GLP-1(7−37)NH2 behave identically in terms of stimulating cAMP production, β-arrestin-1 recruitment and β-arrestin-2 recruitment (Figure S1).

Evaluation of GLP-1R Activation Induced by α/β-Peptides 1−4. As a prelude to evaluating β-arrestin recruitment induced by α/β-peptide analogues of GLP-1, we reassessed cAMP production via a kinetic, GloSensor assay.[41,42] In contrast to previous results for **1−4**,[35] which were based on cAMP accumulation and quantification, cAMP generation potency as monitored by the GloSensor assay declines modestly with each additional α → β replacement (Table 1; Figure 4A). However, the two assays are consistent in indicating that α/β-peptides **1−3** match GLP-1 in terms of maximum cAMP generation via the GLP-1R; the maximum activation by **4** is lower than that of GLP-1 or the other α/β-peptides according to the GloSensor assay.

Although the new assay for cAMP production indicates that α/β-peptide **4** has significantly lower potency and efficacy relative to GLP-1, previous studies demonstrated that **4** can control blood glucose in vivo, and that this effect is prolonged relative to glucose control achieved with GLP-1.[35] In addition, **4** and GLP-1 were indistinguishable as insulin secretagogues in experiments involving mouse pancreatic islets.[35] These functional similarities between GLP-1 and **4** may indicate that the resistance to proteolysis engendered by the nonproteinogenic residues in the α/β-peptide compensates for a diminished efficacy in activating the GLP-1R for cAMP production.

Recruitment of β-arrestin-1 and β-arrestin-2 to the GLP-1R induced by α/β-peptides **1−4** was evaluated via previously described bioluminescence resonance energy transfer (BRET) assays.[16] Constructs for the GLP1R-RLuc8 and GFP²-β-arrestin-1 or GFP²-β-arrestin-2 (R393E, R395E) fusion proteins were transiently transfected into HEK293FT cells along with a construct for G protein-coupled receptor kinase 5 (GRK5). The (R393E, R395E) variant of β-arrestin-2 prevents loss of BRET signal due to GLP-1R internalization.[16] Cotransfection with GRK5 has been shown to enhance the maximum BRET signal in this assay by promoting GLP-1R phosphorylation,[16,22] which increases the affinity of both β-arrestins for the GLP-1R.

Increasing the number of β residues in the GLP-1 analogues, starting near the C-terminus (**1 → 2 → 3**), causes a progressive decline in the potency and/or maximum level of recruitment for each β-arrestin (Table 1, Figures 4B−C). The most dramatic changes are observed for α/β-peptide **4**, which is

Table 1. Potency and Maximum Response Measured for GLP-1 and α/β-Peptides 1−4[a]

	cAMP		β-arrestin-1		β-arrestin-2	
	pEC50 (M)	max response (% GLP-1)	pEC50 (M)	max response (% GLP-1)	pEC50 (M)	max response (% GLP-1)
GLP-1	−10.3 ± 0.1	100	−8.4 ± 0.2	100	−8.1 ± 0.3	100
α/β-peptide **1**	−9.8 ± 0.1	101 ± 1	−8.0 ± 0.1	88 ± 6	−7.9 ± 0.1	78 ± 4
α/β-peptide **2**	−9.4 ± 0.1	100 ± 2	−7.6 ± 0.1	87 ± 6	−7.7 ± 0.1	63 ± 3
α/β-peptide **3**	−8.8 ± 0.1	97 ± 3	−7.8 ± 0.1	48 ± 4	−7.9 ± 0.1	47 ± 4
α/β-peptide **4**	−8.2 ± 0.1	60 ± 5		6 ± 8		13 ± 4

[a]Values are the mean ± SEM of ≥3 independent experiments, with duplicate measurements per experiment. Values represent Gα_s activation, as measured by the luciferase-based GloSensor cAMP reporter assay, β-arrestin-1 recruitment, as measured using a β-arrestin-1 BRET assay and β-arrestin-2 recruitment, as measured using a β-arrestin-2 BRET assay. The β-arrestin-2 plasmid contained (R393E, R395E) mutations, which prevent receptor internalization.[16] For **4**, β-arrestin-1 and β-arrestin-2 maximum responses represent the maximal response at 1 μM peptide.
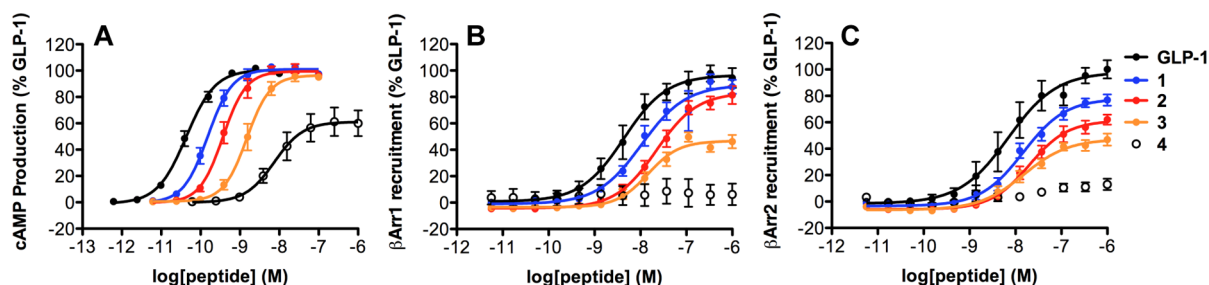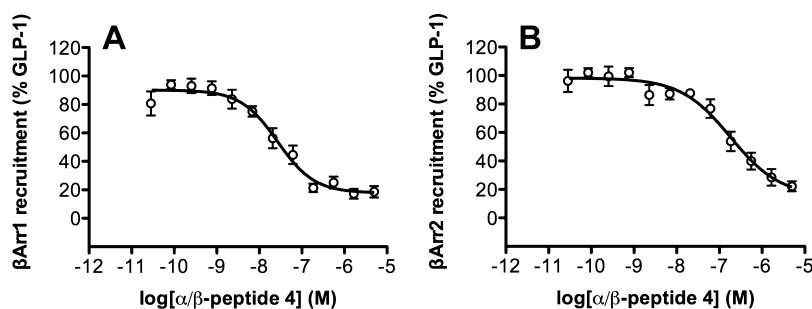
Figure 4. GLP-1R activation comparisons among GLP-1(7−36)NH$_2$ and α/β-peptides **1−4** (based on GLP-1(7−37)NH$_2$). Data points are the mean $\pm$ SEM of $\geq$3 independent experiments, with duplicate measurements for each experiment. Concentration−response curves for peptide-induced activation of GLP-1R as manifested by (A) Gα_s activation, measured by the luciferase-based GloSensor cAMP reporter assay; (B) β-arrestin-1 recruitment, measured using a β-arrestin-1 BRET assay; (C) β-arrestin-2 recruitment, measured using a β-arrestin-2 BRET assay. The β-arrestin-2 plasmid contains (R393E, R395E) mutations, which prevent receptor internalization.[16]

Figure 5. Competition between GLP-1(7−36)NH2 and α/β-peptide **4** for binding to GLP-1R, as detected by BRET assays for recruitment of (A) β-arrestin-1 or (B) β-arrestin-2. Data points are the mean $\pm$ SEM of $\geq$3 independent experiments, with duplicate measurements for each experiment. The concentration of GLP-1(7−36)NH2 was held constant at 10 nM, and the concentration of **4** was varied. The β-arrestin-2 plasmid contains (R393E, R395E) mutations, which prevent receptor internalization.[16]
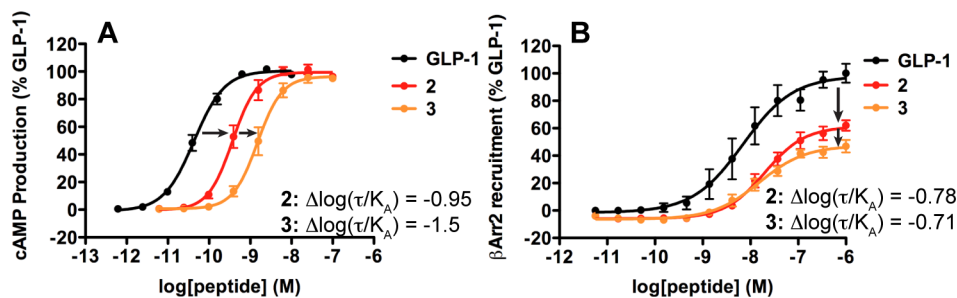

Figure 6. Illustration of the importance of using the operational model for detecting biased agonism. Concentration−response curves generated for GLP-1 and α/β-peptides **2** and **3** in (A) cAMP production and (B) β-arrestin-2 recruitment. The $\Delta\log(\tau/K_A)$ values calculated for α/β-peptide **2** relative to GLP-1 in each response demonstrate that the efficacy of α/β-peptide **2** is similar between cAMP production and β-arrestin-2 recruitment, despite the different changes in the concentration-dependent behavior of this peptide in the two responses. In contrast, the $\Delta\log(\tau/K_A)$ values calculated for α/β-peptide **3** relative to GLP-1 in each response demonstrate that the efficacy of α/β-peptide **3** is greater for β-arrestin-2 recruitment than for cAMP production.

barely active in these assays. α/β-Peptides **3** and **4** have the same five $\alpha \rightarrow \beta$ replacements, but **4** contains two Aib residues not found in **3**.

Competition BRET assays, in which **4** was added to cells in the presence of 10 nM GLP-1, show that this α/β-peptide is fully competent to bind to the receptor under these conditions, because **4** serves as an antagonist of GLP-1-induced recruitment of either β-arrestin-1 (pIC$_{50}$ = −7.6 $\pm$ 0.1) or β-arrestin-2 (pIC$_{50}$= −6.7 $\pm$ 0.2) (Figure 5).

Analysis of GLP-1R Activation Bias by α/β-Peptides 1−4. For a receptor such as GLP-1R that can activate multiple intracellular partner proteins, such as G proteins and β-arrestins, the availability of a new agonist leads to an important question: how do the relative efficacies of this new agonist

among the possible signaling pathways compare with the relative efficacies of a benchmark agonist? In other words, does the new agonist show a preference pattern among the available signaling pathways that differs from the preference pattern of the benchmark agonist? In the context of the present study, a specific example of this type of question is: "how does the relative efficacy of α/β-peptide **3** for inducing cAMP production (presumably via activation of Gα_s) vs recruiting β-arrestin-2 compare with the relative efficacy of the benchmark agonist GLP-1(7−36)NH$_2$ for these two processes?" If the balance of efficacy along these two signaling axes is substantially different for **3** relative to GLP-1(7−36)NH$_2$, then **3** is identified as a "biased agonist" of the GLP-1R.

We have used the operational model of Black and Leff[43,44] to determine whether our α/β-peptide analogues display biased agonism relative to GLP-1(7−36)NH$_2$. This model describes the effect of an agonist on a receptor with two terms, (i) K_A, the dissociation constant that characterizes the agonist binding to the receptor conformation induced by association with a given effector protein (e.g., a G protein or a β-arrestin), and (ii) τ, the efficacy of the agonist, a term that accounts for the potency of agonist action on the receptor and assay-dependent factors, including the density of receptors and strength of receptor-effector coupling.[44] K_A and τ can be estimated from standard concentration−response data sets. The expression $\log(\tau/K_A)$, referred to as the "transduction coefficient," is a measure of the strength of activation of a particular pathway by a specific agonist. The way a given α/β-peptide differs from benchmark agonist GLP-1(7−36)NH$_2$ in activating a particular pathway can be expressed as a difference in transduction coefficients, $\Delta\log(\tau/K_A)$. A "bias factor" for a given α/β-peptide relative to GLP-1(7−36)NH$_2$ can be defined based on comparing two specific outcomes of GLP-1R activation, e.g., cAMP generation vs β-arrestin-2 recruitment, by calculating $\Delta\Delta\log(\tau/K_A)$.

The importance of using the operational model for comparisons among responses involving different effector proteins is illustrated in Figure 6, which focuses on a subset of the data presented in Figure 4. Figure 6A shows that the effect on cAMP production of four or five $\alpha \rightarrow \beta$ replacements in GLP-1, to generate **2** or **3**, is manifested as a rightward shift in the concentration−response curve. There is very little difference among the maxima in cAMP production for these three compounds. In contrast, Figure 6B shows that the effect on β-arrestin-2 recruitment of the same $\alpha \rightarrow \beta$ replacements is manifested primarily as a drop in the maximum recruitment extent. If one focuses on maximum response and pEC$_{50}$, the parameters provided in Table 1, it is not clear how to compare the impact of the $\alpha \rightarrow \beta$ replacements on cAMP production vs β-arrestin-2 recruitment. However, application of the operational model allows calculation of bias factors for **2** and **3** relative to GLP-1 in both assays, and comparison is now straightforward.

Estimation of the $\Delta\Delta\log(\tau/K_A)$ values reveals parallel losses of efficacy for recruitment of β-arrestin-1 and β-arrestin-2 and for cAMP signaling for α/β-peptides **1** and **2** relative to GLP-1(7−36)NH$_2$ (Table 2). For α/β-peptide **3**, however, the loss

Table 2. Bias Factors ($\Delta\Delta\log(\tau/K_A)$) Calculated for GLP-1 and α/β-Peptides 1−3[a]

	$\Delta\Delta\log(\tau/K_A)$	
	β-arrestin-1 vs cAMP	β-arrestin-2 vs cAMP
GLP-1	0	0
α/β-peptide **1**	0.1 ± 0.2	0.1 ± 0.2
α/β-peptide **2**	0.1 ± 0.2	0.2 ± 0.3
α/β-peptide **3**	0.6 ± 0.2	0.8[b] ± 0.2
α/β-peptide **4**	ND	ND

[a]Bias factors ($\Delta\Delta\log(\tau/K_A)$) calculated in terms of β-arrestin-1 recruitment relative to cAMP production, and β-arrestin-2 recruitment relative to cAMP production. These bias factors were derived from experimental data as described in the text. Bias factors could not be calculated for α/β-peptide **4**, because the β-arrestin recruitment assays showed no quantifiable activity for this molecule (Figure 2). ND: not determined. [b]Statistically significant difference from GLP-1 using one-way analysis of variance followed by Dunnett's test ($P < 0.05$).

of cAMP signaling is greater than the loss of β-arrestin-1 or −2 recruitment. Thus, **3** displays a bias for β-arrestin recruitment relative to GLP-1 although the calculated bias factor for **3** is statistically significant only for β-arrestin-2. The lack of statistical significance the for β-arrestin-1 bias factor calculated for **3** may be due to a greater degree of scatter in the β-arrestin-1 BRET data for **1−4** relative to the β-arrestin-2 BRET data for these peptides. Bias factors could not be determined for **4** because no significant recruitment of β-arrestin-1 or -2 could be detected in our assays for this α/β-peptide within the concentration range examined.

Evaluation of Peptides 5−13. New analogues of GLP-1(7−37)NH$_2$ were prepared in an effort to determine whether a particular subset among the substitutions in α/β-peptides **3** and **4** relative to GLP-1 plays a dominant role in mediating biased agonism. α-Peptide analogues containing either the Val16 → Aib replacement (**5**) or both Aib replacements (**6**) were very similar to one another and to GLP-1(7−36)NH$_2$ (Table 3, Figure 7). For the two β-arrestins, both **5** and **6** displayed small declines in maximum recruitment but little change in EC$_{50}$ relative to GLP-1. For cAMP production, both **5** and **6** were ~3-fold less potent than GLP-1 (EC$_{50}$), but both α-peptides were indistinguishable from GLP-1 in terms of maximum response. The similarities in activity among **5**, **6**, and GLP-1(7−36)NH$_2$ suggest that the previously described analogue[38] containing only Ala8 → Aib should manifest an activity profile comparable to those of **5** and **6**.

Analogue **7** contains a single $\alpha \rightarrow \beta$ replacement at position 18; introduction of a β residue at this position in the original series (**2** → **3**) induced an increase in β-arrestin-1 and −2 recruitment bias relative to GLP-1 (Table 2). In contrast, $\alpha \rightarrow \beta$ replacement at position 18 alone causes only minimal (and comparable) changes to cAMP production and β-arrestin recruitment (Table 3, Figure 7). Introduction of additional β residues, at positions 22 and 26 (**8** and **9**), causes decreases in both β-arrestin recruitment and cAMP production relative to GLP-1 (Table 3, Figure 7). Analysis of these data using the operational model (Table 4) indicates that engagement of the GLP-1R by α/β-peptide **8** or **9** causes a preference for recruitment of β-arrestin-1 and -2 over production of cAMP, relative to engagement of the GLP-1R by GLP-1. Thus, α/β-peptides **8** and **9** are β-arrestin-biased agonists of the GLP-1R. α/β-Peptide **10**, containing only the Val16 → Aib and Ser18 → β replacements, displays significant decreases in recruitment of β-arrestin-1 and -2, similar to those seen for **8** and **9**; however, the cAMP response for **10** is stronger than that induced by **8** or **9**, and application of the operational model indicates that α/β-peptide **10** does not show significant signal bias relative to GLP-1.

To define the $\alpha \rightarrow \beta$ replacement sites that are dominant in terms of the bias toward recruitment of β-arrestin-1 and -2 manifested by α/β-peptides **8** and **9**, we prepared and evaluated **11−13** (Table 3, Figure 8). This α/β-peptide set contains each possible pair among the three β residues in **8**. Analysis of the results with the operational model indicates that none among **11−13** displays significant signaling bias relative to GLP-1 (Table 4). Thus, it appears that the three $\alpha \rightarrow \beta$ replacements in **8** represent a minimum level of modification required to generate a β-arrestin-biased agonist for the GLP-1R.

■ DISCUSSION

α/β-Peptide **4** contains seven substitutions relative to GLP-1, which, collectively, lead to losses in this analogue's ability to

Table 3. Potency and Maximum Response Measured for GLP-1 and Peptides 5−13[a]

	cAMP		β-arrestin-1		β-arrestin-2	
	pEC_{50} (M)	max response (% GLP-1)	pEC_{50} (M)	max response (% GLP-1)	pEC_{50} (M)	max response (% GLP-1)
GLP-1	−10.3 ± 0.1	100	−8.0 ± 0.1	100	−8.0 ± 0.1	100
α-peptide 5	−9.8 ± 0.2	101 ± 1	−8.2 ± 0.1	97 ± 2	−8.0 ± 0.1	83 ± 2
α-peptide 6	−9.8 ± 0.1	103 ± 3	−8.1 ± 0.1	86 ± 4	−7.9 ± 0.1	76 ± 4
α/β-peptide 7	−10.0 ± 0.1	99 ± 1	−7.9 ± 0.1	81 ± 5	−8.1 ± 0.1	84 ± 2
α/β-peptide 8	−9.0 ± 0.2	96 ± 1	−7.7 ± 0.2	43 ± 3	−8.0 ± 0.1	63 ± 5
α/β-peptide 9	−8.4 ± 0.2	97 ± 2	−7.5 ± 0.1	41 ± 4	−8.0 ± 0.1	59 ± 2
α/β-peptide 10	−9.6 ± 0.1	100 ± 1	−8.0 ± 0.1	30 ± 1	−8.0 ± 0.1	53 ± 5
α/β-peptide 11	−9.8 ± 0.1	102 ± 1	−8.0 ± 0.1	37 ± 1	−8.1 ± 0.1	52 ± 4
α/β-peptide 12	−10.0 ± 0.1	103 ± 1	−7.8 ± 0.1	85 ± 3	−8.0 ± 0.1	78 ± 3
α/β-peptide 13	−10.1 ± 0.1	104 ± 1	−7.8 ± 0.2	80 ± 3	−8.0 ± 0.1	74 ± 4

[a]Values are the mean ± SEM of ≥3 independent experiments, with duplicate measurements for each experiment. Values represent $G\alpha_s$ activation, as measured by the luciferase-based GloSensor cAMP reporter assay, β-arrestin-1 recruitment, as measured using a β-arrestin-1 BRET assay and β-arrestin-2 recruitment, as measured using a β-arrestin-2 BRET assay. β-Arrestin-2 plasmid contained (R393E, R395E) mutations, which prevent receptor internalization.[16]
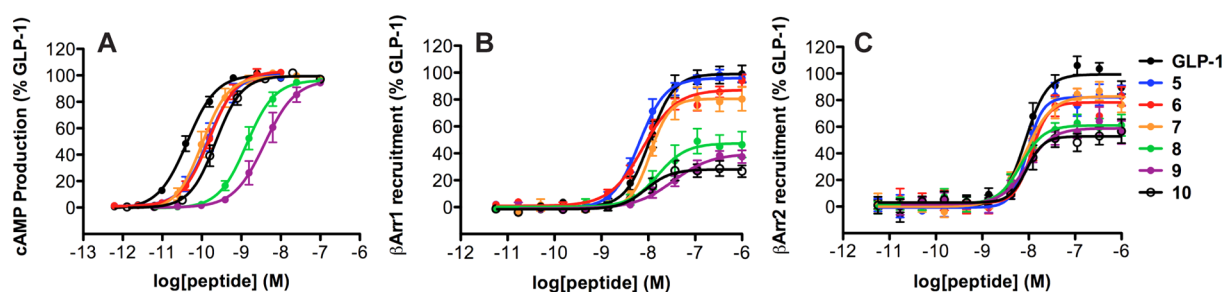
Figure 7. GLP-1R activation comparisons among GLP-1(7−36)NH₂ and α/β-peptides 5−10 (based on GLP-1(7−37)NH₂). Data points are the mean ± SEM of ≥3 independent experiments, with duplicate measurements for each experiment. Concentration−response curves for peptide-induced activation of GLP-1R as manifested by (A) $G\alpha_s$ activation, measured by the luciferase-based GloSensor cAMP reporter assay; (B) β-arrestin-1 recruitment, measured using a β-arrestin-1 BRET assay; (C) β-arrestin-2 recruitment, measured using a β-arrestin-2 BRET assay. The β-arrestin-2 plasmid contains (R393E, R395E) mutations, which prevent receptor internalization.[16]

Table 4. Bias Factors ($\Delta\Delta\log(\tau/K_A)$) Calculated for GLP-1 and Peptides 5−13[a]

	$\Delta\Delta\log(\tau/K_A)$	
	β-arrestin-1 vs cAMP	β-arrestin-2 vs cAMP
GLP-1	0	0
α-peptide 5	0.2 ± 0.1	0.2 ± 0.1
α-peptide 6	0.2 ± 0.1	0.0 ± 0.1
α/β-peptide 7	0.2 ± 0.1	0.2 ± 0.1
α/β-peptide 8	0.6[b] ± 0.2	1.2[b] ± 0.1
α/β-peptide 9	0.9[b] ± 0.2	1.6[b] ± 0.1
α/β-peptide 10	0.0 ± 0.1	0.3 ± 0.1
α/β-peptide 11	0.1 ± 0.1	0.2 ± 0.1
α/β-peptide 12	−0.0 ± 0.1	0.1 ± 0.1
α/β-peptide 13	−0.3 ± 0.2	−0.1 ± 0.1

[a]Bias factors ($\Delta\Delta\log(\tau/K_A)$) calculated in terms of β-arrestin-1 recruitment relative to cAMP production, and β-arrestin-2 recruitment relative to cAMP production. These bias factors were derived from experimental data as described in the text. [b]Statistically significant difference from GLP-1 using one-way analysis of variance followed by Dunnett's test ($P < 0.05$).

stimulate cAMP production or induce β-arrestin recruitment, relative to GLP-1. Exploration of subsets among the α → β and α → Aib replacement sites of 4 reveals substitution patterns that have little effect on peptide efficacy in terms of either cAMP production or β-arrestin recruitment (5, 6, 12, and 13), substitution patterns that reduce peptide efficacy in each

signaling pathway (1, 2, 7, 10, and 11) and, most interestingly, substitution patterns that differentially affect cAMP production versus β-arrestin recruitment (3, 8, and 9).

Comparing 3, 8, and 9 indicates that α → β replacements in the central region of GLP-1 are the most consequential in terms of engendering a bias toward β-arrestin recruitment relative to cAMP production. While the incorporation of an α → β replacement at position 18 (2 → 3) is necessary for the induction of selectivity for β-arrestin recruitment, neither the Ser18 → β substitution alone (7) nor double substitution at Ser18 and Gly22 (11) is sufficient to induce this selectivity. In addition, implementing only the two outer α → β replacements in 8, at Ser18 and Lys26, to generate 13, does not induce pathway selectivity. These findings are significant because they suggest that G protein activation is more sensitive than is β-arrestin recruitment to changes in a relatively focused region near the middle of a GLP-1R agonist. In addition, our results suggest that this region of the peptide must contain a minimum density of α → β replacements (e.g., all three α → β replacements in 8) in order to induce a bias toward β-arrestin recruitment relative to G protein-mediated signaling.

The importance revealed here of the GLP-1 segment encompassing residues 18−26 in terms of $G\alpha_s$ activation by the GLP-1R, as manifested by cAMP production, can be related to the available structural information for the hormone-GPCR complex. A cocrystal structure of GLP-1(7−37) with the extracellular domain of GLP-1R[37] shows that direct contact begins at hormone residue Ala24 and extends toward the
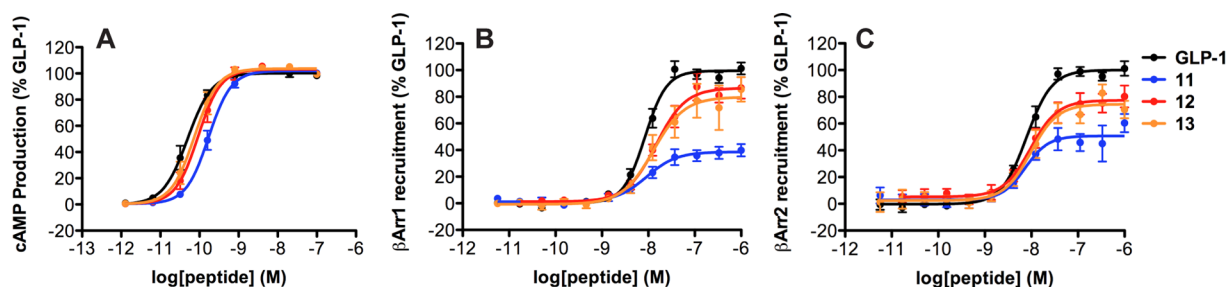
Figure 8. GLP-1R activation comparisons among GLP-1(7−36)NH₂ and α/β-peptides **11−13** (based on GLP-1(7−37)NH₂). Data points are the mean ± SEM of ≥3 independent experiments, with duplicate measurements for each experiment. Concentration−response curves for peptide-induced activation of GLP-1R as manifested by (A) Gα_s activation, measured by the luciferase-based GloSensor cAMP reporter assay; (B) β-arrestin-1 recruitment, measured using a β-arrestin-1 BRET assay; (C) β-arrestin-2 recruitment, measured using a β-arrestin-2 BRET assay. The β-arrestin-2 plasmid contains (R393E, R395E) mutations, which prevent receptor internalization.[16]

hormone C-terminus; thus, interactions of the 18−26 region of GLP-1 with the receptor must largely involve the membrane-embedded domain of the GLP-1R. Photoreactive labeling previously identified a direct interaction between position 20 of GLP-1 and Trp-297 in extracellular loop 2 (ECL2) of the GLP-1R.[45] Receptor mutation studies revealed that ECL2 plays an important role in GLP-1-induced signaling (cAMP production, Ca^{2+} mobilization and ERK phosphorylation).[9,11] In addition, recent mutation studies involving ECL3 suggest that several residues in this loop are critical in mediating signaling induced by GLP-1, exendin-4 or oxyntomodulin.[26] The α → β replacements made in the 18−26 region of GLP-1 to generate **8** may disrupt critical interactions between the ligand and the ECLs of the GLP-1R.

To begin to explore how β-arrestin-biased α/β-peptides might interact with the ECLs of the GLP-1R, we examined a recently reported model of the GLP-1 + GLP-1R complex, and highlighted the positions of GLP-1 that are modified in α/β-peptide **8** to generate Figure 9.[46] This image suggests that there
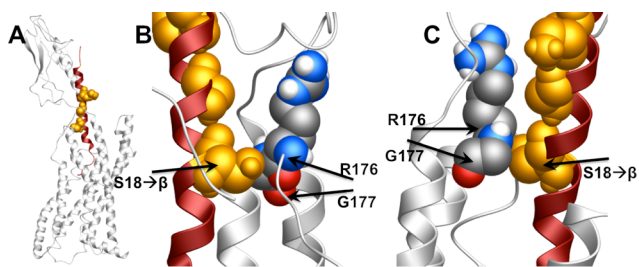

Figure 9. Images depicting a hypothetical interaction between the GLP-1R (gray) and α/β-peptide **8** (red), generated from an existing model of the GLP-1R complexed to GLP-1,[43] by highlighting in orange the residues in GLP-1 that are modified to β residues in **8**. (A) Full view of the GLP-1R + GLP-1 model. (B, C) Close-up views of position 18 of GLP-1 (β residue in **8**) in contact with R376 and G377 of ECL3 of GLP-1R. Images were generated using MolSoft ICM Browser software.

may be a close contact between the β residue at position 18 of **8** and residues within ECL3 that were previously found to be critical in mediating ERK1/2 phosphorylation induced by GLP-1, exendin-4 and oxyntomodulin via their interactions with the GLP-1R.[26] Oxyntomodulin is biased toward ERK1/2 phosphorylation over cAMP production relative to GLP-1, a selectivity that may arise from the bias of oxyntomodulin toward β-arrestin recruitment.[26] Based on this precedent, we speculate that the contacts we propose between the central

portion of **8** and ECL3 of the GLP-1R might contribute to the β-arrestin bias displayed by this α/β-peptide.

As noted above, **8** and related α/β-peptides have lower efficacy relative to GLP-1 in terms of cAMP production and β-arrestin recruitment, but the impact of α → β substitution on cAMP production is larger than the impact on β-arrestin recruitment. The results of the computational modeling raise the possibility that interactions between centrally located β residues on these α/β-peptides and ECL3 on the receptor may explain why efficacy for β-arrestin recruitment suffers less from α → β replacement than does efficacy for cAMP production.

■ CONCLUSIONS

We have shown that evaluation of a small family of peptide hormone derivatives containing α → β replacements can lead to discovery of agonists with significant signaling bias relative to the native hormone, despite the decline in overall signaling efficacy among the α/β-peptides relative to GLP-1 itself. This type of backbone-modified peptide is readily accessible via conventional solid-phase synthesis but has received relatively little attention from the perspective of exploring new agonists for Class B GPCRs. Promising results have recently been reported for α/β analogues of parathyroid hormone(1−34)[47] and vasoactive intestinal peptide.[48] Collectively, these recent studies and the work reported here suggest that backbone-modified analogues of peptide hormones may prove to be a rich source of new agonists that can serve as tools for elucidating the physiological consequences of specific GPCR-initiated signaling pathways; these unusual peptides may ultimately lead to therapeutic advances.

Our finding that a central segment of GLP-1 appears to be more critical for G protein-mediated activity than for β-arrestin recruitment at the GLP-1R is of general significance from the perspective of new strategies for discovery of biased agonists of this medically important receptor. In addition, this finding is intriguing in the context of the recent report of a G protein-biased analogue of exendin-4.[27] Exendin-4 is a lizard-derived peptide that serves as a potent GLP-1R agonist and is approved for treatment of type 2 diabetes.[23] The new α-peptide was identified via screening of a massive biosynthetic library of exendin-4 variants differing in the N-terminal segment.[27] Thus, the bias induced by this combinatorially derived peptide arises from changes at the N-terminus of exendin-4, an observation that contrasts with and is complemented by our finding that β-arrestin bias can be achieved via modifications in the central segment of GLP-1.

EXPERIMENTAL METHODS

Peptide Synthesis and Purification. Peptides were prepared by microwave-assisted solid phase peptide synthesis[36] on ChemMatrix H-PAL amide resin based on Fmoc-protection of the main chain amino groups. Full details on peptide synthesis and purification can be found in the Supporting Information.

cAMP Production Assays. cAMP signaling was assessed in HEK293-derived cells (GS-22A)[39] stably expressing the GloSensor cAMP reporter.[40] These cells were a gift from Prof. Thomas Gardella at Massachusetts General Hospital. Human GLP-1R was transiently transfected into these cells (10 μg GLP-1R for 10 cm plate of cells) using FuGene HD transfection reagent (3:1 FuGene HD to GLP-1R). On the day of transfection, culture medium was replaced with antibiotic-free DMEM supplemented with 10% FBS, 4 mM L-glutamine, and 1 mM sodium pyruvate. GLP-1R and FuGene HD were combined in OptiMEM (1 mL), and this mixture was incubated for 15 min before being added to cells. Cells were exposed to transfection reagents for 24 h before being plated into opaque, clear-bottomed 96-well plates at 30 000 cells per well in antibiotic-free DMEM supplemented with 10% FBS, 4 mM L-glutamine, and 1 mM sodium pyruvate. The plate was incubated for 24 h. For cAMP concentration−response assays, cells were preincubated with D-PBS buffer containing D-luciferin (0.5 mM) until a stable background luminescence signal was achieved (30 min). Various doses of peptide were then added, and luminescence was measured for 30 min on a BioTek Synergy 2 plate reader. The maximal luminescence response (usually observed 16−20 min after peptide addition) was used to generate concentration−response curves.

Reported EC_{50} and maximum response values are the average of ≥3 independent experiments. Each experiment involved ≥7 different concentrations of GLP-1 or analogue, with solutions prepared via serial dilution of a stock solution of each peptide (usually 1 μM, which becomes 100 nM in the assay), with each resulting data point representing the average of two replicate wells. Changing pipet tips after each dilution was found to be critical for reproducible concentration−response curves. Both commercially obtained GLP-1 and GLP-1 we had prepared were tested in these assays, with identical results (Figure S2). GLP-1(7−36)NH$_2$ and GLP-1(7−37)NH$_2$ behaved identically in these assays (Figure S1).

Bioluminescence Resonance Energy Transfer Assays. Recruitment of β-arrestin-1 and β-arrestin-2 to the GLP-1R was assessed in HEK293FT cells, using a bioluminescence resonance energy transfer (BRET) assay based on a previous report.[16] GLP-1R-Rluc8, GFP^2-β-arrestin-1, *or* GFP^2-β-arrestin-2(R393E, R395E), and GRK5 (which has previously been shown to enhance BRET signal in this assay[22]) were transiently cotransfected using polyethylenimine (PEI). A 1:1 ratio of PEI/DNA was used, because cell viability was compromised at higher PEI/DNA ratios. On the day of transfection, culture medium was changed to DMEM with no supplements. DNA and PEI were combined in OptiMEM, and the mixture was incubated at room temperature for 20 min before being added to cells. After 6 h, DMEM supplemented with 20% FBS was added to the cells. After 24 h of transfection, cells were plated into opaque, white-bottomed 96-well plates and incubated for 24 h.

Prior to BRET experiments, cells were rinsed twice with PBS and then incubated with D-PBS for 1 h at 37 °C. Various concentrations of peptide were then added and incubated for 20 min at 37 °C. Rluc8 substrate DeepBlueC (60 μM in EtOH) was then added and incubated for 20−40 min before BRET signal (I_{515nm}/I_{410nm}) was measured on a Tecan Infinite 200 or BioTek Synergy 2 plate reader. Incubation with DeepBlueC prior to signal detection reduced signal variability, which presumably arises in the absence of preincubation because of variability in time required for DeepBlueC to penetrate cells and become available for oxidation by Rluc8. The BRET signal for GLP-1 usually remained constant over this time, though sometimes the signal for GLP-1 and analogues decreased slightly (to similar extents) over the course of 1 h. In some experiments, we observed that the BRET signal for some α/β-peptides (7 and 10) to decrease slightly (~5% decrease relative to GLP-1) over the course of an hour. Thus, the BRET signal

for each peptide was measured at the same time in replicate experiments.

Reported EC_{50} and maximum response values are the average of ≥3 independent experiments. Each experiment consists of ≥7 different concentrations of GLP-1 or analogue, with solutions prepared via serial dilution of the stock solution of each peptide (usually 12 μM, which becomes 1 μM in the assay), with each resulting data point representing the average of two replicate wells.

For competition BRET assays, various concentrations of α/β-peptide **4** were added to cells, and a constant concentration of GLP-1 (130 nM, which becomes 10 nM in the assay) was added immediately after addition of α/β-peptide. All subsequent steps were performed as above.

Full details on molecular biology operations that were employed to access BRET fusion protein constructs and on BRET transfection ratio optimization can be found in the Supporting Information.

Data Analysis. Data were processed using Microscoft Excel and GraphPad Prism 5 software. Concentration−response data for each experiment were normalized to the maximum signal observed for GLP-1(7−36)NH$_2$ for that experiment. The normalized data points from each independent experiment were averaged to determine the average concentration−response behavior for GLP-1 and each analogue as depicted in concentration−response curves. EC_{50} and maximum response values were extracted from concentration−response data by fitting the concentration−response for each individual experiment to a sigmoidal dose−response model with variable slope, and then calculating the average and SEM for each individual experiment.

To quantify the efficacy of each peptide in the different pathways assayed, each concentration−response curve for each pathway (cAMP production, β-arrestin-1 recruitment, and β-arrestin-2 recruitment) was fitted to an operational model of agonism,[43,44] as shown in eq 1. In eq 1, [A] is the concentration of agonist and E is the response at each tested [A] (i.e., the independent and dependent variables in the assay). As described in the main text, K_A is the dissociation constant that characterizes the agonist binding to the receptor conformation induced by association with a given effector protein. τ is the efficacy of the agonist, a term that accounts for the potency of agonist action on the receptor and assay-dependent factors, including the density of receptors and strength of receptor−effector coupling.

$$E = \frac{E_{max}\tau[A]}{[A](1+\tau) + K_A} \tag{1}$$

For each peptide, the efficacy in each signaling pathway was extracted as the $\log(\tau/K_A)$ (mean ± SEM), and then compared to GLP-1 by subtracting the value of $\log(\tau/K_A)$ for GLP-1 from that of the peptide to yield $\Delta\log(\tau/K_A)$ for the peptide. The $\Delta\log(\tau/K_A)$ values obtained for the peptide relative to GLP-1 were complied to determine the average ± SEM $\Delta\log(\tau/K_A)$ for the peptide. The bias factor (differential efficacy of a peptide relative to GLP-1 in two separate pathways) for each peptide was determined by subtracting the $\Delta\log(\tau/K_A)$ in the arrestin pathway from the $\Delta\log(\tau/K_A)$ for that peptide in the cAMP pathway, yielding $\Delta\Delta\log(\tau/K_A)$ for each peptide. Error in each $\Delta\Delta\log(\tau/K_A)$ value was calculated by propagating the error in the $\log(\tau/K_A)$ (SEM obtained from averaging individual $\Delta\log(\tau/K_A)$ values) for the peptide and GLP-1 in each pathway (cAMP and either β-arrestin-1 recruitment or β-arrestin-2 recruitment).

To determine whether the $\Delta\Delta\log(\tau/K_A)$ for a given peptide was statistically significant, $\Delta\Delta\log(\tau/K_A)$ values were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post test. $P < 0.05$ denotes statistical significance.

ASSOCIATED CONTENT

⑤ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08323.

Additional figures and details for experimental protocols (PDF)

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Notes

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