Differential Requirement of the Extracellular Domain in Activation of Class B G Protein-coupled Receptors*

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G protein-coupled receptors (GPCRs) from the secretin-like (class B) family are key players in hormonal homeostasis and are important drug targets for the treatment of metabolic disorders and neuronal diseases. They consist of a large N-terminal extracellular domain (ECD) and a transmembrane domain (TMD) with the GPCR signature of seven transmembrane helices. Class B GPCRs are activated by peptide hormones with their C termini bound to the receptor ECD and their N termini bound to the TMD. It is thought that the ECD functions as an affinity trap to bind and localize the hormone to the receptor. This in turn would allow the hormone N terminus to insert into the TMD and induce conformational changes of the TMD to activate downstream signaling. In contrast to this prevailing model, we demonstrate that human class B GPCRs vary widely in their requirement of the ECD for activation. In one group, represented by corticotrophin-releasing factor receptor 1 (CRF1R), parathyroid hormone receptor (PAC1R), and pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC1R), the ECD requirement for high affinity hormone binding can be bypassed by induced proximity and mass action effects, whereas in the other group, represented by glucagon receptor (GCGR) and glucagon-like peptide-1 receptor (GLP-1R), the ECD is required for signaling even when the hormone is covalently linked to the TMD. Furthermore, the activation of GLP-1R by small molecules that interact with the intracellular side of the receptor is dependent on the presence of its ECD, suggesting a direct role of the ECD in GLP-1R activation.

The class B or secretin family of GPCRs consists of 15 receptors for peptide hormones that include glucagon, glucagon-like peptides, parathyroid hormone, and calcitonin (Fig. 1A). These receptors are important drug targets for many human diseases including diabetes, neurodegeneration, cardiovascular diseases, and psychiatric disorders. The full-length receptors consist of two modular domains: a globular extracellular domain (ECD) defined by three conserved disulfide bonds and a TMD that contains seven transmembrane helices (1–4). The ECD is responsible for the high affinity and specificity of hormone binding, and the TMD is required for receptor activation and signal coupling to downstream G proteins and other signaling effectors (4). Peptide hormone binding is thought to proceed through fast binding of its C terminus to the ECD followed by a slower association of the peptide N terminus with the receptor TMD (5), which leads to conformational changes in the receptor TMD and the receptor activation. The activated receptors are coupled primarily to stimulatory G proteins, resulting in elevation of the intracellular cAMP level.

Structures of the ECDs of class B GPCRs in complex with their peptide ligands have been determined by x-ray crystallography (1, 6–9) and NMR (10) and have provided useful information about structural mechanisms of ligand recognition and selectivity (2, 11). Only recently, crystal structures of the iso-

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The abbreviations used are: ECD, extracellular domain; TMD, transmembrane domain; GPCR, G protein-coupled receptors; GCG, glucagon; GCGR, GCG receptor; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; PTH, parathyroid hormone; PTH1R, PTH1 receptor; CRF, corticotropin-releasing factor; CRF-R, CRF receptor 1; PAC1R, pituitary adenylate cyclase activating polypeptide type 1 receptor; CRE, cAMP-response element; BETP, 4-(3-(benzoyloxy)phenyl)-2-(ethylsulfanyl)-6-(trifluoromethyl)pyrimidine; ICL3, intracellular loop 3; RLU, renilla luciferase activity.
lated TMDs of two class B receptors in an inactive conformation have been published, i.e. the human glucagon receptor (GCGR) (12, 13) and human corticotropin-releasing factor receptor 1 (CRF1R) (14). Although crystal structures of more than 20 class A GPCRs have been determined, currently no full-length structure of a class B receptor has been reported due to the inherent instability of these receptors and the limited availability of stabilizing high affinity small molecule agonists and antagonists.

Although the TMDs of class B GPCRs are highly homologous, homology of the ECDs is limited to the six disulfide-forming cysteines and only about a dozen other conserved residues. Most ECD structures have been obtained only in complex with ligands, indicating that ligand binding stabilizes these proteins and, therefore, favors crystallization of ECD-ligand complexes. In contrast to endogenous peptide hormones, small molecule antagonists, such as CP-376395 (a CRF1R antagonist), which were discovered by high throughput screening, act allosterically within the TMD to block the binding and signaling of the peptide agonist ligands (14, 15).

In this report we demonstrate that the ECDs of some class B GPCRs, such as CRF1R, PAC1R, and PTH1R, solely function as affinity traps, and hence, their requirement can be bypassed by mass action effects and hormone tethering, consistent with the prevailing model of peptide binding and activation of Class B GPCRs. In contrast to this prevailing model, activation of GCGR and GLP-1R is strictly dependent on the presence of their ECDs. We further provide comprehensive insights into the mechanism for the requirement of ECD in activation of GCGR and GLP-1R by small molecule ligands and by muta-
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Results

Class B GPCRs Differ in Their ECD Requirements for Signaling—To examine the requirement of the GCGR ECD for signaling, we transiently transfected four different FLAG-tagged constructs into HEK293 cells: the full-length receptor, TMD, TMD-interacting N terminus of glucagon (1–15) fused to the TMD, and a fusion in which glucagon (1–15) and the TMD are separated by a thermostabilized cytochrome b562 variant, BRIL, which has been used for facilitating GPCR crystallization (16) (Fig. 1, B–D). If the ECD only functions as an affinity trap to increase the local concentration of the glucagon N terminus, we reasoned that a high concentration of exogenous hormone may bypass the requirement for the ECD. We, therefore, studied the activation of a cAMP-responsive CRE-luciferase reporter to monitor GCGR signaling in the presence or absence of 500 nM exogenous peptides; n = 3, error bars = S.D.

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Concentration in blood is ~25 pm (17, 18). As shown in Fig. 1, C and D, the TMD was well expressed but remained inactive under either condition. As expected, full-length GCGR was active in...

**FIGURE 4.** Membrane-anchored GCG and GLP-1 constitutively activate their full-length receptors but not their TMD. A, schematic depiction of the experiment. B and C, fusion of the C termini of GCG (B) and GLP-1 (C) to a single membrane-spanning helix (GCG-M and GLP-1-M) constitutively activate their corresponding full-length receptors but not their corresponding TMDs; n = 3, error bars = S.D.

**FIGURE 5.** Concentration curves of GCG (A) and GLP-1 (B) in activation of their full-length and TMD receptors. For the full-length (FL) receptor, the EC50 value for GCG is 3.4 nM and the EC50 value for GLP-1 is 7.0 nM. For TMD, there is no activation up to 10 μM GCG or GLP-1. All data are presented as cAMP signal (RLU); n = 3, error bars = S.D.

**FIGURE 6.** Mutational analysis of the GCG-GCGR fusion protein. cAMP reporter activity of transfected expression constructs that have mutations within the GCG part (A) or the GCGR part (B and C) of the fusion protein; n = 3, error bars = S.D. Residues substituted with alanine in B are known GCG-interacting residues; residues labeled by an asterisk are located on a discrete surface shown in Fig. 8.
the presence, but not the absence, of the hormone. Although pioneering work by the Vale laboratory demonstrated that directly fusing CRF to the CRF1RΔECD generated a constitutively active receptor (19), a chimeric fusion between glucagon and the GCGR TMD with or without BRIL linker was inactive (Fig. 1C).

Next, we extended this analysis to four other members of class B GPCRs; GLP-1R, PTH1R, CRF1R, and PAC1R. GLP-1R behaved in a manner similar to GCGR in that activity required both the full-length receptor and free peptide hormone (Fig. 1E), even though the GLP-1R TMD and GLP-1-TMD fusions were well expressed (Fig. 1F). In contrast, the ECDs for PTH1R, CRF1R, and PAC1R were not required in the presence of high concentrations of their respective hormones, and their requirements could also be bypassed by directly fusing the N-terminal, TMD-interacting hormone fragments to their respective TMDs (Fig. 2), resulting in constitutive receptor activation. The results of the chimeric receptor experiments are in agreement with previous reports on CRF1R, CRF1RΔ, and PAC1R (20), consistent with the prevailing model that the ECD is only required for high affinity binding but not for activation of these receptors. Fusion of glucagon and GLP-1 to their full-length receptors, separated by 5 repeats of a Gly-Ser-Ala linker (15 amino acid linker = GSA₅), constitutively activated the CRE-luciferase reporter (Fig. 3), indicating that fused glucagon and GLP-1 in the presence of their respective ECD can productively interact with the TMD.

Membrane tethering of glucagon and GLP-1 has been shown as another way to engineer proximity between receptor and peptide hormone and to bypass the requirement for exogenous hormone (19). In this approach proximity occurs by membrane co-localization, i.e. without directly fusing hormone and receptor and thereby avoiding potential steric constraints impeding the interaction between covalently linked binding partners. In these experiments, we fused glucagon and GLP-1 to a long flexible linker and the single membrane-spanning helix of CD8 (20) and co-expressed these chimeric peptides with both the GCGR and GLP-1R TMDs as well as full-length receptors. Consistent with the previous report (20), membrane-anchored glucagon and GLP-1 constitutively activated their full-length receptors (Fig. 4). However, they failed to stimulate signaling through GCGR and GLP-1R lacking respective ECDs (Fig. 4). Collectively, we conclude that class B GPCRs vary in the extent of their ECD requirements for receptor activation and that the receptors can be divided into two functionally distinct subgroups: one group including PTH1R, CRF1R, and PAC1R, in which the ECD only functions as affinity trap and is, therefore, dispensable when the affinity requirement is bypassed, and the other group including GCGR and GLP-1R that requires the ECD for receptor activation. This conclusion is further supported by
full-concentration curves of GCG and GLP-1 to their respective receptors (Fig. 5). Both GCG and GLP-1 activate their respective full-length receptor at low nM concentrations, but they cannot activate their TMDs lacking the ECD even at 10 μM of peptides.

Activity-compromising Mutations in the GCGR ECD Cluster at a Surface Distant from Hormone Binding—To understand the ECD requirement for activation of GCGR, we carried out mutational studies of the ECD and the fused hormone peptide in the context of the GCG-GSA5-GCGR fusion protein. We first mutated residues in the glucagon part of the protein. Tyr-10 and Tyr-13 in the N terminus of glucagon have been predicted to make key interactions with the GCGR TMD (12). Their replacements with arginine residues (Y10R and Y13R) strongly reduced activity of the hybrid receptor, but activity could be fully recovered in the presence of 500 nM exogenous glucagon (Fig. 6A). This indicates that the intramolecular interaction with the fused mutant peptide can be efficiently displaced by exogenous native peptide, suggesting that the glucagon-ECD interaction is an important contributor for the overall affinity of glucagon-GCGR binding and activation. Replacement of Trp-25 in the ECD-interacting C terminus of the fused glucagon had a partial signaling defect even though the fusion positions the hormone in the vicinity of the TMD independent of an ECD interaction.

We subsequently introduced 27 single amino acid alterations into the fusion proteins that collectively span the entire length of the ECD: W36A, D63A, P86S, F31R, E34R, K37R, L38R, D41R, H45R, L49R, L56R, N74R, T76R, N78R, W83R, H89R, V91L, F95R, F97R, R99E, D103R, V107R, P114R, W115R, D117N, S119R, and Q120R (Fig. 6, B and C). Three groups of mutant proteins stood out. The first group of mutations...
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**Small Molecule GLP-1R Modulators Mimic Endogenous Peptide Hormones or Modify the GLP-1R Intracellular Loop 3—** Several non-peptidic GLP-1R agonists were identified by high throughput screening and subsequent structural optimization (24–26). Interestingly, none of them appears to simply bind the GLP-1R TMD. One set of compounds, exemplified by the large substituted cyclobutanes Boc5 (27), S4P (27), and WB4–24 (28, 29) (Fig. 10A) competed with the ECD binding antagonist exendin(9–39) (an N-terminally truncated, non-TMD interacting version of the Gila Monster GLP-1R agonist extendin-4; Ref. 30) indicating that they directly bind the ECD. In addition, Boc5 and S4P largely, but not completely, displaced labeled GLP-1 from GLP-1R and may, therefore, also interact with the TMD (27). In contrast, the pyrimidine BETP (31) was not competed by exendin(9–39) or GLP-1 and is, therefore, thought to allosterically activate GLP-1R (31–34). Recent work has demonstrated that BETP functions by forming covalent adducts with Cys-347 in the intracellular loop 3 (ICL3) of GLP-1R, which may mimic a physiological covalent modification (35).

We tested these compounds for their ability to stimulate either full-length GLP-1R or the GLP-1R TMD. All compounds stimulated cAMP production in cells transfected with full-length GLP-1R, and no compound, even up to 100 μM concentration, could activate signaling in the absence of the ECD (Figs. 10B and 11). Moreover, when we mutated the GLP-1R residue that becomes covalently modified by BETP, Cys-347, to alanine, BETP almost completely lost its ability to activate the full-length GLP-1R, whereas activation by GLP-1 was unaffected (Fig. 10C). Collectively, this shows that BETP activates GLP-1R by a mechanism that is both markedly different and independent of activation by the native peptide and that may involve binding and covalent modification of ICL3. Despite these distinct mechanisms, BETP activation of GLP-1R is still dependent on the intact GLP-1R ECD.

**Discussion**

Biochemical, mutational, and structural experiments have provided evidence for a two-domain, two-step model of class B GPCR activation (4). This model posits that the C termini of peptide hormones bind the receptor ECDs to bring the peptide N termini into the vicinity of the receptor TMDs, which then allows the N termini to bind a pocket inside of the TMD. The latter is thought to induce conformational changes that are propagated to the intracellular side of the receptor TMD to allow coupling to heterotrimeric G proteins to stimulate the intracellular signaling cascade. In the simplest version of the model, the ECD would only function as affinity trap, and its included Trp-36, Asp-63, and Pro-86, which were previously identified as glucagon binding residues in a structure model of the full-length glucagon-GCGR complex (12), and their mutations abrogated glucagon binding (12, 21). The corresponding fusion proteins were expressed at levels comparable with those of the wild type fusion protein (Fig. 7) but almost abolished receptor activation both in the presence or absence of exogenous added glucagon (Fig. 6B). Second, seven well expressed mutant proteins (N74R, T76R, W83R, V91L, F95R, F97R, D103R Q120R; marked with asterisks in Fig. 6C) had activities that were reduced by ≥2-fold compared with the wild type yet whose activity defect could be rescued by 500 nM exogenous glucagon. We mapped the corresponding residues on the crystal structure of the structurally highly homologous GLP-1R ECD in complex with GLP-1 (6) (PDB code 3IOL; highlighted in yellow in Fig. 8, A and B). Surprisingly, none of these residues was in bonding distance to the peptide hormone, and none was on the long α1-helix, which is thought to interact with the TMD under basal conditions but not in the presence of glucagon (22, 23). Instead, all of them were exposed on the third accessible surface of the complex (the other two surfaces are formed by the hormone and the α1-helix). Therefore, a substantial set of residues required for GCGR activity did not appear to be involved in ligand binding but likely formed an inter- or intra-molecular interaction surface. Third, six mutations mapped to the α1-helix (E34R, K37R, L38R, D41R, H45R, and L49R; Figs. 6C and 8) and showed moderately higher activity than the wild type fusion protein, except for L49R, which displayed the same activity as the wild type. This observation is consistent with the proposed inhibitory effect of the α1-helix on basal GCGR activity (22, 23). To explore functional conservation, we also analyzed the effects of 10 corresponding GLP-1/GLP-1R mutants in the context of the GLP-1/GLP-1R fusion protein. As seen in Fig. 9, these mutations had similar, but less pronounced effects, especially in the absence of exogenous GLP-1.

**FIGURE 9. Activity and expression levels of GLP-1R ECD mutant proteins.**

A, cAMP activity of GLP-1(7–36)-FLAG-GSAR-GLP-1R fusion receptors; n = 3, error bars = S.D. B, expression levels of the fusion receptors determined by FACS (top) and by immunoblotting (bottom), both with anti-FLAG antibody.
requirement for receptor activation could thus be bypassed by mass action or tethering.

In this study we have demonstrated that class B GPCRs differ in their requirement for the ECD in receptor activation. As shown in Fig. 12, one group of receptors, represented by CRF1R, PAC1R, and PTH1R, can be activated without ECD when peptide is presented in high concentrations (Fig. 12A) or fused to the TMD (Fig. 12B). In contrast, the other group, represented by GCGR and GLP-1R, requires the ECD for activation even in the presence of a very high concentration of native peptide or when the peptide hormones were artificially tethered to the TMD or to the membrane (Figs. 1, 3, and 4). It is unlikely that this requirement is due to an inherent lack of hormone affinity for the GCGR and GLP-1R TMDs. Rather, our mutational analysis of glucagon in this study and quantitative binding assays of GLP-1/glucagon chimeric hormones in a previous study (36) indicate that both hormones bind the TMDs of their respective receptors.

In addition to the endogenous peptide hormones, the small molecule GLP-1R agonists Boc5, S4P, and WB4–24 also directly bind the receptor ECD (27, 28) and require the ECD for signaling (Fig. 10), consistent with a critical role of the ECD in mediating conformational changes in the TMD and intracellular loops. In contrast, BETP, a small molecule GLP-1R modulator that does not bind the ECD, signals through a distinct mechanism that appears to function in parallel to GLP-1 (Refs. 32, 34, and 35) and that induces a distinct (“biased”) downstream signaling response (34). Surprisingly, the ability of BETP but not GLP-1 to stimulate GLP-1R signaling depended on Cys-347 in ICL3 (Fig. 10C), the same residue that has been shown to

FIGURE 10. Differential stimulation of human GLP-1R and GLP-1R TMD signaling by small molecule GLP-1R activators. A. Structures of BETP, Boc5, S4P, and WB4–24. B. cAMP reporter activities of the indicated transfected expression constructs in the absence or presence of 1 μM GLP-1 (control) or 10 μM concentrations of the indicated small molecule activators; n = 3, error bars, S.D. C. Activation of wild type (WT) and C347A GLP-1R was by BETP and GLP1; n = 3, error bars = S.D.
be covalently modified by BETP. These results suggest that the ECD played a direct role in the activation of GLP-1R, even by the small molecule agonists that act on the intracellular side of the receptor.

The inability to bypass the tethering function of the GCGR and GLP-1R ECDs implies a mechanism that hormone binding to the ECDs of GCGR and GLP-1R directly affects the ECD-TMD interaction (Fig. 12C), which could contribute to conformational changes and activation of the receptors. Strong evidence has been presented for an intramolecular ECD-TMD interaction in GCGR that is regulated by glucagon (22, 23). It has been proposed that the \(\alpha_1\)-helix of the ECD interacts with the extended first transmembrane helix (the TM1 stalk) and the extracellular loops of the TMD to auto-inhibit GCGR activity under basal conditions and that this interaction is disrupted by glucagon binding (22, 23). Consistent with this model, we observed that mutations in \(\alpha_1\) residues mildly increase GCGR activity (Fig. 6C). However, we unexpectedly found that activity-compromising mutations in residues that are not involved in ligand binding and that do not affect the level of membrane-bound receptor all localize to a contiguous surface of the ECD. This surface faces away from hormone and TMD and thus may form an interface for a currently unidentified interaction that is important for receptor activation. We speculate that this proposed ECD-TMD interface (Fig. 12C) could serve as a possible mechanism for the requirement of the ECD for activation of GCGR and GLP-1R. In addition, our results presented in this paper provide a basis for re-classifying class B GPCRs based on their differential requirement of the ECDs for receptor activation.

**Experimental Procedures**

*Reagents*—Restriction enzymes were purchased from Fermentas. DNA polymerase was obtained from New England Biolabs. DMEM/high glucose and fetal bovine serum (FBS) were from HyClone and Gibco. Trypsin-EDTA, phosphate-buffered saline (PBS), GlutaMAX, HEPES, sodium pyruvate, and sodium butyrate were procured from Gibco. Doxycycline was from Sigma.

*Peptide Synthesis*—Human PTH(1–34), glucagon(1–29), GLP-1(7–36), PACAP(1–27), and CRF(1–41) peptides for cAMP assays were custom-synthesized and HPLC-purified by GL Biochem (Shanghai) Ltd. Peptides were dissolved and diluted in H2O.

*Small Molecule GLP-1R Modulators*—Boc5, S4P, WB4–24 and pyrimidine BETP were synthesized in-house with purities >95% using published procedures (37, 38). These compounds were dissolved in 100% DMSO and diluted in buffer (final DMSO concentration was 1%) and added at 10 \(\mu M\) final com-

![Concentration curves of small molecules in activation of full-length human GLP-1R and TMD.](image-url)
Plasmid Construction—Codon-optimized cDNAs of human class B GPCRs lacking the predicted signal peptide coding region were synthesized by Genewiz (CRF₁R (residues 23–384), PAC1R (residues 22–422), PTH1R (residues 27–486), GCGR (residues 26–431), and GLP-1R (residues 24–463)). The encoded fragments were digested with BamHI and NotI restriction endonucleases and inserted into a modified pcDNA6 expression vector to encode a fusion protein consisting of an N-terminal human IgG leader (MGWSCIILFLVATATGVHSE) for targeting into the cell membrane and a FLAG tag (DYKDDDD) at the C terminus for detection by immunoblotting. In addition to the full-length receptors, we generated the same set of constructs with (i) the TMDs of GCGR (residues 123–431), GLP-1R (residues 140–463), PTH1R (residues 182–484), CRF₁R (residues 110–384), and PAC1R (residues 148–421), (ii) fusions between the N-terminal peptide hormones (glucagon (1–15), GLP-1(7–21), PTH(1–15), UNC(1–15), PACAP(1–12)) and their corresponding TMDs, (iii) fusions of the same N-terminal peptide fragments as above to the BRIL-TMD constructs, and (iv) fusions of the full-length peptide hormones to the N terminus of their corresponding full-length receptors with five copies of Gly-Ser-Ala (GSA₅) linker. All these constructs contain the same IgG leader as above. The TMD and full-length PAC1R constructs were N-terminally tagged with two copies of maltose-binding protein, which was used for affinity tag purification.

Mutagenesis—Thirty single amino acid mutations were made in the GCG-GCGR fusion receptor, including W36A, D63A, P86S, F31R, E34R, K37R, L38R, D41R, H45R, L49R, L56R, N74R, T76R, N78R, W83R, H89R, V91L, F95R, F97R, R99E, D103R, V107R, P114R, W115R, D117N, S119R, and Q120R, which collectively span the entire length of the ECD, and Y10R, Y13R, and W25R, which are residues in the fused glucagon. Site-directed mutagenesis experiments were carried out using the QuikChange method (Agilent). Mutations and all plasmid constructs were confirmed by DNA sequencing.
Cell Culture—A HEK293 suspension cell line was cultured in DMEM/high glucose medium with L-glutamine (HyClone) supplemented with 10% FBS (Gibco). Cells were incubated at 37 °C and supplemented with 5% CO₂ in a humidified chamber. When cells reached 80–90% confluence they were detached by trypsinization and reseded by 4–6-fold dilution into fresh medium. They were also seeded in 24-well plates when needed, and plasmids were transfected into the cells. Protein expression was induced by adding doxycycline and sodium butyrate at 1 μg/ml and 10 mM, respectively. After induction of protein expression, cells were incubated for 18–24 h until collected for further use.

Transfection and Luciferase Assays—Cells were plated at a density of 5 × 10⁴ per well in a 24-well plate and incubated overnight at 37 °C. Cells were then transiently transfected using Lipofectamine reagent (Life Technologies) with expression plasmids of a CRE-driven fly luciferase reporter (200 ng) and a TK promoter-driven renilla luciferase (10 ng), which was used as an internal transfection control. Three hours after transfection, the cells were treated with 1 μM doxycycline for 24 h, then peptides or ligands were added to cells for a 4-h incubation.

After 4-h incubation with peptides or small molecule GLP-1R modulators, cells were harvested and lysed in Passive Lysis Buffer (Promega). The cAMP concentration in lysate was measured sequentially from a single sample. All experiments (RLU) and the second measurement (Renilla luciferase) were normalized to luciferase activity. The first measurement (firefly luciferase activity of CRE-driven fly luciferase reporter relative to renilla luciferase activity) was used to normalize data to an internal transfection control. Three hours after transfection, the cells were treated with 1 μM doxycycline for 24 h, then peptides or ligands were added to cells for a 4-h incubation.

Membrane-tethered Ligands—Codon-optimized sequences encoding glucagon(1–29) and GLP-1(7–36), were synthesized by GENEWIZ. These sequences were cloned in a modified pcDNA6 expression vector that contains an IgG leader (MGWSCIILFLVATATGVHSE) followed by the peptide hormone, a MYC tag (EQKLISEEDL), a NG5 linker (GNGNGNGNGNGNGNGNG), a single pass TMD (ALCWVGLGVLAAGVLVVTAIVYVV) with a 3XFLAG tag at the C terminus of the fusion constructs. These constructs followed the same design as the published membrane-anchored PTH (19). IgG leader is the signal peptide that guides the secretory protein into the cell membrane. Constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen).

Western Blots—Cells were harvested by centrifugation, and their pellets were solubilized in cell lysis reagent (CellLytic™ M, Sigma) supplemented with 1 mM PMSF and centrifuged at 16,100 × g for 30 min. The supernatants were subjected to reducing and non-reducing SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and incubated with anti-FLAG M2 antibodies produced in mouse (Sigma) or monoclonal anti-β-actin antibody produced in mouse clone AC-15 (Sigma) followed by HRP-conjugated anti-mouse antibodies in 5% milk dissolved in TBST. Protein bands were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and images were collected using a ChemiDoc™ XRS+ imager (Bio-Rad).

FACS Analysis—To prepare samples for FACS, the cells were washed twice with PBS resuspended in PBS containing 0.1% BSA and incubated for 15 min on ice with monoclonal anti-FLAG® M2-FITC antibody produced in mouse (Sigma) (1 μg per 10⁶ cells), and samples were analyzed using a BD Accuri™ C6 Flow Cytometer instrument using Accuri C6 software (BD Biosciences).

Statistical Analysis—GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA) was used to fit data to a 3-parameter dose-response curve. All data are presented as the mean ± S.D.

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