Published in final edited form as:

J Am Chem Soc. 2017 September 27; 139(38): 13376–13386. doi:10.1021/jacs.7b05674.

Spirohexene-Tetrazine Ligation Enables Bioorthogonal Labeling of Class B G Protein-Coupled Receptors in Live Cells

Carlo P. Ramil[†], Maoqing Dong[‡], Peng An[†], Tracey M. Lewandowski[†], Zhipeng Yu^{†,‡}, Laurence J. Miller^{‡,*}, and Qing Lin^{†,*}

[†]Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260-3000, United States

[‡]Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, Arizona 85259, United States

Abstract

A new bioorthogonal reactant pair, spiro[2.3]hex-1-ene (Sph) and 3,6-di-(2-pyridyl)-*s*-tetrazine (DpTz), for the strain-promoted inverse electron-demand Diels-Alder cycloaddition, i.e., tetrazine ligation, is reported. Compared with the previously reported strained alkenes such as *trans*-cyclooctene (TCO) and 1,3-disubstituted cyclopropene, Sph exhibits balanced reactivity and stability in tetrazine ligation with the protein substrates. A lysine derivative of Sph, SphK, was site-selectively incorporated into the extracellular loop regions (ECLs) of GCGR and GLP-1R, two members of class B G protein-coupled receptors (GPCRs) in mammalian cells with the incorporation efficiency dependent on the location. Subsequent bioorthogonal reactions with the fluorophore-conjugated DpTz reagents afforded the fluorescently labeled GCGR and GLP-1R ECL mutants with labeling yield as high as 68%. A multitude of functional assays were performed with these GPCR mutants, including ligand binding, ligand-induced receptor internalization, and ligand-stimulated intracellular cAMP accumulation. Several positions in the ECL3s of GCGR and GLP-1R were identified that tolerate SphK mutagenesis and subsequent bioorthogonal labeling. The generation of functional, fluorescently labeled ECL3 mutants of GCGR and GLP-1R should allow biophysical studies of conformation dynamics of this important class of GPCRs in their native environment in live cells.

Graphical abstract

*Corresponding Authors: qinglin@buffalo.edu, miller@mayo.edu.

[#]Present Address

Sichuan University College of Chemistry, Chengdu, China

Supporting Information

Supplemental figures, tables and methods, synthetic schemes, characterization of new compounds and proteins, movie of GCGR internalization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

The class B family of G protein-coupled receptors (GPCRs) comprises 15 peptide-binding receptors in human, including the receptors for secretin, glucagon (GCG), glucagon-like peptide 1 (GLP-1), corticotropin-releasing factor, parathyroid hormone, and the calcitonin gene-related peptide. These receptors are validated drug targets for the treatment of many diseases including diabetes, depression, and osteoporosis.^{1,2} From structural standpoint, class B GPCRs share ~15% sequence homology with the larger class A GPCRs. A distinctive feature of the class B receptors is that they invariably contain a large N-terminal extracellular domain (ECD, typically 100–160 residues) in addition to the prototypical seven-transmembrane domain (TMD). Recently, the crystal structures of TMDs of two class B GPCRs, the glucagon receptor (GCGR)³ and corticotropin-releasing factor 1 receptor (CRF1R),⁴ were reported, revealing a wider and deeper cavity in their ligand-binding cores than their class A counterparts.

Despite rapid progress in structural elucidation of non-rhodopsin GPCRs,⁵ our understanding of GPCR dynamics, especially the mechanism by which ligands induce specific conformational change in live cells, remains limited.⁶ A major hurdle in biophysical studies of GPCR activation in live cells is that there are very few techniques available that allow site-specific introduction of biophysical probes *in vivo* without negatively affecting receptor function.⁷ So far, biophysical studies of the class B GPCRs have focused on mapping peptide ligand-receptor interactions using the fluorescently labeled peptide ligands,⁸ disulfide trapping,⁹ cross-linking with *p*-benzoyl-L-phenylalanine-modified peptide ligands¹⁰ and the genetically encoded receptors containing fluoromethylketone¹¹ and azidophenylalanine.¹² While these studies have increased our understanding of the ligand-receptor interactions,¹³ it is still desirable to install a fluorophore *directly* into the conformational mobile region of the class B GPCRs so that we can monitor the ligand-induced receptor conformational transition in real time in live cells.

To introduce a fluorophore into the middle part of a membrane protein, e.g., a flexible loop, with minimum perturbation to protein function in live cells, three strategies have been successfully developed, including: (i) insertion of a short peptide tag that can be selectively functionalized by enzymes¹⁴ or chemical reagents¹⁵; (ii) site-specific incorporation of a fluorescent amino acid via genetic code expansion;¹⁶ and (iii) site-specific incorporation of a synthetic amino acid carrying a chemical reporter followed by selective bioorthogonal reactions in a two-step process referred to as bioorthogonal labeling.¹⁷ Among these approaches, bioorthogonal labeling is most versatile as the use of a chemical reporter minimizes structural perturbation while at the same time allows a quick access to a palette of

bright fluorescent dyes.¹⁸ Indeed, a growing repertoire of bioorthogonal reactions¹⁹ in conjunction with a myriad range of genetically encoded unnatural amino acids carrying unique functionalities has opened up new venues of protein functional studies in situ in living cells.²⁰ Bioorthogonal labeling has been successfully employed in fluorescent labeling of the purified or reconstituted class A GPCRs such as rhodopsin,²¹ CCR5,²² and ghrelin receptor²³ carrying the azide reporter via the strain-promoted azide-alkyne cycloaddition reaction with the cyclooctyne reagents.

We previously reported the design and synthesis of a strained spirocyclic alkene, spiro[2.3]hex-1-ene (Sph) for an accelerated tetrazole-based photoclick chemistry.²⁴ For substituted cyclopropene derivatives, it was postulated that 3,3-disubstituted cyclopropenes are good reactants for the photoclick chemistry while 1,3-disubstituted cyclopropenes are good reactants for the tetrazine ligation, and that their reactivities are mutually exclusive.²⁵ Since Sph is a 3,3-disubstituted cyclopropene, albeit a double strained one, we decided to investigate its reactivity in the tetrazine ligation reaction and its suitability as a chemical reporter for bioorthogonal labeling of GCGR and GLP-1R, two members of the class B GPCRs implicated in diabetes and obesity. Herein, we report the comparison studies of the reactivity of spirohexene (Sph) vs. *trans*-cyclooctene (TCO) in the tetrazine ligation with the 3,6-di-(2-pyridyl)-s-tetrazine (DpTz) reagents both in vitro and in live cells. We then show that this new Sph-DpTz reactant pair enabled fast bioorthogonal labeling of GCGR and GLP-1R bearing the genetically encoded Sph-lysine at their respective extracellular loop 3 (ECL3) region in live cells. A number of sites within the ECL3 of these receptors encoding Sph underwent bioorthogonal labeling in live cells with high efficiency. The resulting fluorescently labeled receptors were functional based on the intracellular cAMP accumulation assay and exhibited ligand and time-dependent receptor internalization.

Results and Discussion

Reactivity Comparison of Sph vs. TCO and Cyclopropene

The TCO–tetrazine ligation is the fastest bioorthogonal reaction known today with the second-order rate constant approaching $10^6 \text{ M}^{-1} \text{ s}^{-1}$.²⁶ This fast kinetics makes it particularly valuable for applications involving low substrate abundance such as membrane GPCRs. The use of TCO as a chemical reporter, however, is not without limitation. For example, TCO is known to undergo *trans*→*cis* isomerization in the presence of thiols or copper-containing proteins,²⁷ with the resulting *cis*-cyclooctene 100,000-fold less reactive.²⁸ To overcome this limitation, alternative strained cycloalkenes such as cyclopropene (Cyp) have been developed with improved stability; however, the Cyp derivatives exhibit significantly slower reaction kinetics.²⁹ For GPCR substrates with only a few thousand copies on the cell membrane, a robust alkene reporter with balanced reactivity and stability is imperative. To this end, the kinetics of the ligation reaction between superfolder green fluorescent protein (sfGFP) encoding a strained alkene at position-204 and DpTz-TAMRA (**6**) was measured *via* FRET by following the decrease in sfGFP fluorescence in phosphate buffer, pH 7.4. We found that Sph-lysine (SphK, **3** in Chart 1) encoded sfGFP gave a second-order rate constant (k_2) of $7,900 \pm 1,000 \text{ M}^{-1} \text{ s}^{-1}$, while the corresponding TCOK (**4** in Chart 1, single axial diastereomer) encoded sfGFP gave a k_2 value of $38,000 \pm 7,000 \text{ M}^{-1}$

s⁻¹, approximately 5 times faster than sfGFP-204SphK (Figure S1 in SI), similar to what was reported previously.³⁰ By comparison, the CypK-encoded sfGFP have a much lower k_2 value of $280 \pm 10 \text{ M}^{-1} \text{ s}^{-1}$ (Figure S1 in SI), indicating that the doubly strained SphK is 28-fold more reactive than CypK in the tetrazine ligation reaction.

Reactivity Comparison Using Strained Alkene-Labeled SNAP-Tag

To further compare the reactivity of Sph with other strained alkenes on proteins, we employed the SNAP-tag,³¹ a 20-kDa mutant of *O*⁶-alkylguanine-DNA alkyltransferase that reacts specifically with the strained alkene-tethered benzylguanine (BG-alkene; Step 1 in Figure 1a). The reactivity of the resulting strained alkene-labeled SNAP-tag (SNAP-alkene) can then be directly assessed by incubating SNAP-alkene with DpTz-FL (**7**, Step 2 in Figure 1a). In-gel fluorescence analysis showed that Sph exhibits a reactivity similar to TCO but greater than cyclopropene (Figure 1b). LC-MS analyses revealed that SNAP-Sph gave quantitative conversion after 10-min incubation, whereas SNAP-TCO (single axial diastereomer) gave 86% conversion (Figure 1c and Figure S2), which could be attributed to partial isomerization of TCO to the more stable *cis*-form because of the presence of 1 mM DTT in the reaction buffer. In comparison, SNAP-Cyp gave 52% conversion under the same condition (Figure 1c and Figure S2), indicating markedly lower reactivity.³² To examine their relative stability, we incubated 20 mM each of TCO (**2**) and SphK (**3**) with 50 mM glutathione in a deuterated acetonitrile/PBS buffer mixture (1:1) and monitored their decay by ¹H NMR over a period of 5 days. The half-life ($t_{1/2}$) was determined to be 41 hours for SphK and 23 hours for TCO (Figure S3), indicating that Sph is more stable than TCO in the presence of thiols. The LC-MS analysis showed that a major decomposition pathway of SphK appears to be glutathione addition to SphK (Figure S3d).

To probe whether the Sph-tetrazine ligation is suitable for protein labeling in live cells, we fused SNAP-tag to the N-terminus of GLP-1R and expressed SNAP-GLP-1R in HEK293T cells. We then treated cells expressing SNAP-GLP-1R with BG-alkenes **11–13** followed by reactions with DpTz-FL (Figure 1d). The expression of SNAP-GLP-1R in HEK293T cells was confirmed by strong fluorescence at the plasma membrane after treating cells with BG-FL (Figure 1e). Both BG-TCO and BG-Sph-treated cells showed robust labeling whereas BG-Cyp-treated cells showed weaker fluorescence; the labeling is specific as the BG-amine-treated cells did not show fluorescence (Figure 1e).

Identifying Suitable GCGR Sites for UAA Mutagenesis

While unnatural amino acids such as *p*-2'-fluoroacetylphenylalanine¹¹ and *p*-azidophenylalanine¹³ have been incorporated site-selectively into various positions of corticotropin-releasing factor receptor type 1 (CRF1R) via genetic code expansion, it remains to be determined what positions in GCGR and GLP-1R can tolerate UAA mutagenesis. To this end, we tested the incorporation of *N*^ε-Boc-L-lysine (BocK), an excellent substrate for wild-type *Methanosarcina mazei* pyrrolysyl-tRNA synthetase (*MmPylRS*),³³ at position-55 of the ECD as well as 6 positions in ECL3 of the TMD using GCGR-GFP as a reporter in HEK293T cells. Confocal micrographs revealed that *MmPylRS* can charge BocK into all seven positions (Figure S4), with expression levels ranging from 13% for D370BocK to 39% for H372BocK relative to the wild-type based on GFP

fluorescence intensity (Figure 2a). In the absence of BocK, the amber mutants of GCGR-GFP were expressed at much lower levels in a range of 1–13% of the wild-type depending on the positions (Figure 2a). This apparent amber codon read-through can be attributed to the incorporation of endogenous amino acids into these positions by the wild-type PylRS. Indeed, the incorporation of endogenous amino acids in GLP-1R amber mutants by a mutant tyrosyl-tRNA synthetase in mammalian cells was also observed in a recent study.¹²

To assess the effect of BocK mutagenesis on GCGR biological activity, we measured the ability of the mutant to respond to glucagon stimulation using an intracellular cAMP accumulation assay (Figure 2b and Table S1). The E55BocK mutant showed an activity similar to the wild-type (pEC_{50} = 10.2 vs. 10.9 for wild-type), consistent with the fact that E55 is located outside of the glucagon binding pocket. Among the ECL3 mutants, GCGR-H372BocK showed the highest potency with pEC_{50} value of 9.9 and E_{max} of 65% of the wild-type (Table S1). This is in agreement with the GCGR 7-transmembrane helical bundle structure in which H372 projects away from the proposed ligand binding site and is solvent exposed.³ In the absence of BocK, all amber mutants except E55, H372 and Q374 showed very low levels of cAMP accumulation (Figure 2b and Table S1).

Our initial studies verified that SphK can be site-specifically incorporated into superfolder green fluorescent protein and epidermal growth factor receptor in HEK293T cells with an excellent efficiency (Figure S5–S6). We then proceeded to introduce the alkene reporters into the same positions of GCGR and examined whether the resulting GCGR mutants were functional. We found that in general SphK exhibited higher incorporation efficiency than TCOK into GCGR-GFP in HEK293T cells (Figure S7). The more efficient utilization of SphK as an alkene reporter in GCGR is likely a result of superior substrate properties toward its cognate synthetase. To monitor GCGR activity, we turned to the ligand-induced receptor internalization assay as it represents a key mechanism for terminating GCGR signaling following receptor activation.^{34,35} Accordingly, we prepared a Cy5-labeled glucagon peptide, GCG-Cy5, and examined whether GCG-Cy5 treatment of HEK293T cells expressing the SphK-encoded GCGR leads to internalization of the mutant receptors. Both wild-type and two selected mutants, E55SphK and H372SphK, underwent the ligand-induced internalization based on confocal microscopy (Figure 2c), indicating that these SphK-encoded GCGR mutants are functional.

Bioorthogonal Labeling of GCGR in Live Cells

Selective reactions with the alkene-containing proteins in live cells via tetrazine ligation has proven to be a robust method for labeling biomolecules in living systems.³⁶ To introduce a fluorophore into GCGR via tetrazine ligation, we first compared the labeling efficiency and specificity of SphK vs. TCOK as a chemical reporter in live cells by treating HEK293T cells expressing the alkene-encoded GCGR-GFP mutants with DpTz-AF647 (**8**, Chart 1) and visualizing the AF647-labeled GCGR-GFP products by confocal microscopy. Both SphK- and TCOK-encoded cells, but not BocK-encoded cells, showed specific labeling in which the AF647 signals co-localized with the GFP at the plasma membrane (Figure 3a and Figure S8–S10). In all positions, SphK exhibited higher labeling efficiency than TCOK (Figure S8 and S9). For example, the GCGR-GFP-D370TAG mutant can only participate in SphK-

mediated bioorthogonal labeling, but not TCOK (Figure 3a). Since D370 is located at the end of TM6 close to the plasma membrane (Figure 3b), it is possible that the genetically encoded TCO moiety may insert into the plasma membrane, making it inaccessible to the DpTz-AF647 reagent.

To estimate bioorthogonal labeling yield of the SphK-encoded GCGR mutant, we first measured the cell surface expression levels of the wild-type, GCGR-H372SphK and SNAP-GCGR by fluorescence-activated cell sorting (FACS) using rabbit anti-GCGR antibody and allophycocyanin (APC)-conjugated anti-rabbit IgG(Ab')₂ fragment. It is noted that HEK293 cells do not show a detectable amount of endogenous GCGR expression as measured by its mRNA level.³⁷ Relative to the wild-type, GCGR-H372SphK was expressed at 39% while SNAP-GCGR was expressed at 152% (Figure 3c). We then treated SNAP-GCGR with excess BG-Cy3 and set the yield of SNAP-mediated labeling of SNAP-GCGR at 100%. By comparing the fluorescence intensity of GCGR-H372SphK after tetrazine ligation with DpTz-Cy3 to that of SNAP-GCGR-Cy3, the Sph-tetrazine ligation yield was estimated to be 68%, after correcting their difference in expression level (Figure 3c).

Functional Characterization of Fluorescently Labeled GCGRs

Depending on the incorporation site, amino acid side chain structure and its posttranslational modification, it is generally very difficult to predict the effect of bioorthogonal labeling on protein function. To probe whether the fluorescently labeled GCGR mutants retain their function, we examined their ligand binding properties using confocal microscopy. A series of Cy3-labeled GCGR mutants were obtained by treating cells expressing intact GCGR encoding SphK at various positions with DpTz-Cy3 (9, Chart 1). The binding of GCG-Cy5 to the Cy3-labeled GCGR mutants was confirmed on the basis of co-localization of Cy3 and Cy5 signals; among six ECL3 mutants, H372 showed highest activity based on Cy5 fluorescence intensity (Figure 4a), suggesting that an appropriately positioned Cy3-labeled GCGR mutant retains its ability to bind the ligand.

To probe whether the Cy3-labeled GCGR mutant shows similar cAMP response, we measured the activities of GCGR-H372Cy3 and GCGR-H372SphK in the cAMP assay and found that the presence of the Cy3 label at position-372 did not affect the receptor activity (Figure 4b). However, compared to the wild-type, the SphK mutagenesis led to 10-fold lower potency ($pEC_{50} = 9.6$ vs. 10.7 for WT) with essentially no change in maximum response (Table 1). In the absence of SphK, a significantly lower receptor activity was detected ($E_{max} = 35\%$ of the wild-type). This apparent amber codon read-through is attributed principally to a low activity of *Mm*PylRS in charging endogenous amino acids as the control experiment with cells expressing GCGR-H372TAG in the absence of *Mm*PylRS/tRNA_{CUA} and SphK with 12% maximum response relative to the wild-type (Figure 4b and Table 1). Furthermore, to measure the internalization kinetics of the glucagon-activated GCGR, we followed the glucagon-induced localization change of the Cy3-labeled GCGR-H372 mutant by confocal microscopy over a period of 60 min. We observed steady increase of the intracellular fluorescence signals over this period with the maximal intensity reached at 60 min (Figure 4c). This kinetic behavior is very similar to the one reported with SNAP-GCGR in a TR-FRET assay.³⁵ A separate time-lapsed imaging experiment with a single

HEK293T cell displaying the Cy3-labeled GCGR-H372 mutant further verified time-dependent, glucagon-induced receptor internalization (Figure S11 and supplemental movie). Together, the data indicate that the site-specifically fluorescently labeled GCGR mutants are fully functional.

Bioorthogonal Labeling of GLP-1R and Functional Characterization

With the Sph-tetrazine ligation fully validated with GCGR, we extended this reaction to the labeling of GLP-1R, a member of the class B GPCR family that shares high sequence and structural homology with GCGR. To begin, we introduced amber mutation into 11 positions of GLP-1R-GFP, including D59 in the ECD, and 10 residues in the TMD (4 in ECL2: E294, G295, T298, and S301; and 6 in ECL3: E373, H374, A375, R376, G377, and T378), and estimated the BocK incorporation efficiencies based on GFP fluorescence. Unlike GCGR, GLP-1R appears less tolerant to BocK mutagenesis with only five mutants expressing at a level greater than 10% of wild-type (Figure 5a). Among four ECL2 mutants, only E294 expressed at 32% of the wild-type. Aside from low amber suppression efficiency, all ECL2 mutants showed poor responses in GLP-1-induced cAMP accumulation assay with $E_{\max} < 5\%$ of the wild-type (Figure 5b, Table S2), suggesting a critical role of ECL2 in ligand binding and receptor activation.³⁸ On the other hand, the ECL3 is more permissive of BocK mutagenesis as three ECL3 mutants (H374, G377, and T378) showed significant cAMP accumulation with $E_{\max}$ 18% of the wild-type (Figure 5b, Table S2). It was not well understood whether the ECL3 of GLP-1R is directly involved in receptor activation.³⁹ Our data suggest that ECL3 is not as important as ECL2 in ligand binding and receptor activation. In the absence of BocK, low levels of receptor expression were detected, typically in the range of 1–7% (Figure 5a). Importantly, none of the amber mutants showed significant cAMP activity (Figure 5b, Table S2). Since ECL3 is more permissive of UAA mutagenesis, we generated the SphK-encoded intact GLP-1R ECL3 mutants and labeled them with DpTz-FL via the Sph-tetrazine ligation. Confocal microscopy revealed that only three mutants encoding SphK at A375, R376, or T378 showed significant labeling (Figure 5c). Interestingly, the labeling efficiency does not follow the same order as the incorporation efficiency (Figure 5a), suggesting that SphK is likely more accessible at these positions. To examine whether the fluorescently labeled GLP-1R remains active, cells expressing the Cy3-labeled GLP-1R-T378SphK mutant were stimulated with 1 μ M of GLP-1 peptide. Substantial internalization of the GLP-1R mutant was observed after 15-min stimulation (Figure 5d). Importantly, cells expressing the fluorescently labeled GLP-1R mutant did not lose activity in the cAMP accumulation assay (Figure 5e and Table 2), indicating that addition of Cy3 fluorophore on ECL3 is well tolerated. In the absence of SphK, the background amber suppression at position-378 gave greatly diminished cAMP activity (Figure 5e and Table 2).

While the conceptual framework for site-specific, bioorthogonal protein labeling is well established, the application of this approach to the class B GPCRs has not been reported. In this work, we found that the effect of bioorthogonal labeling of class B GPCRs on receptor function is rather complex and difficult to predict *a priori*. This inherent complexity may arise from the interplay of several factors. First, there appears a positional effect in charging unnatural amino acids *via* amber codon suppression. For example, the incorporation

efficiency of BocK into GLP-1R varies significantly, ranging from 1% at position-301 to 68% at position-59, relative to the wild-type (Figure 5a). While the mechanism of how the sequence context of amber codon affects suppression efficiency remains unclear, this type of sequence dependency has been previously observed in *E. coli*.⁴⁰ In principle, the amber suppression efficiency in GCGR and GLP-1R can be further improved using known approaches such as the optimization of PylT expression,⁴¹ the inclusion of a nuclear export signal in the PylRS construct,⁴² and the use of a baculovirus vector for more efficient delivery of the PylRS/PylT pair.⁴³

Another complication may arise from accessibility of the strained alkene, depending on its chemical structure and its location on the receptor. TCOK appears to be less solvent-exposed, particularly when it is located close to a hydrophobic environment, e.g., plasma membrane (Figure 3b). As a result, TCOK exhibits reduced reactivity toward the tetrazine reagents. In contrast, despite its lower intrinsic reactivity (Figure S1), SphK gave greater labeling efficiency with the same tetrazine reagents due to its apparently higher accessibility (Figure 3a). In this context, the Sph-tetrazine reactant pair represents a superior pair for bioorthogonal labeling where the chemical reporter exists in or close to a hydrophobic environment. The attenuation of TCO reactivity in a hydrophobic environment has been previously observed in the TCO-mediated antibody-small molecule conjugation studies.⁴⁴

Of note, we did not observe a correlation between the incorporation efficiency and the function of the UAA-encoded class B GPCRs. For example, position-375 of GLP-1R charges BocK at a higher level (Figure 5a); however, despite its high expression, this mutant exhibits very low receptor activity (Figure 5b). Therefore, it is imperative that the biological activities of the UAA mutants are assessed independently in order to identify functional mutants for in vivo biophysical studies. It is equally important to re-assess the biological activity after bioorthogonal labeling (Figure 4b and 5e) to verify that the posttranslational modification does not alter receptor function.

Taken together, it is evident that the generation of functional, fluorescently labeled GPCRs requires a concerted approach involving simultaneous multi-parameter optimization. The success of this effort is critically dependent on the identification of suitable positions for the incorporation of the chemical reporter, the selection of a reactant pair that is stable, solvent-accessible, and yet sufficiently reactive, and independent testing of the receptor mutants carrying the fluorescent side chain to ensure functional integrity. Toward this end, the Sph/DpTz reactant pair appears to offer the right balance of stability, accessibility, and reactivity in this complex system.

In summary, we have identified a new bioorthogonal reactant pair, Sph and DpTz, for the strain-promoted inverse electron-demand Diels-Alder cycloaddition reaction. When Sph is introduced into proteins site-selectively through either a substrate for SNAP enzyme, i.e., BG-Sph, or genetic encoding in the form of Sph-lysine, it displays a balance of reactivity and stability both in vitro and in cultured cells. Using this new reactant pair, we demonstrate for the first time site-specific introduction of a fluorophore into the ECL regions of GCGR and GLP-1R carrying the genetically encoded SphK through bioorthogonal tetrazine ligation in live cells. Furthermore, by subjecting the resulting ECL receptor mutants to a series of

functional assays, we identified functional, fluorescently labeled ECL3 mutants of GCGR and GLP-1R that are potentially useful in the study of class B GPCR dynamics. During the preparation of this manuscript, the full-length structures of GCGR⁴⁵ and GLP-1R^{46,47} were reported, offering an exciting opportunity to probe receptor dynamics using the fluorescently labeled receptors such as those reported herein, in native cellular environment. Finally, it should be possible to install two fluorescent probes, one in the ECD and one in ECL3, by combining this strategy with other labeling schemes such as SNAP-tag, to sense the domain movement during ligand stimulation.

Experimental Section

HEK293T cell culture and transfection

HEK293T cells were maintained in growth medium containing Dulbecco's modified eagle medium (DMEM, Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies) and 10 µg/mL Gentamicin (Life Technologies). Transfection was performed at 70–80% confluency using 3:1 reagent:DNA ratio of Lipofectamine 2000 (Life Technologies) with 2.5 µg total DNA or polyethylenimine (PEI, Polysciences, Inc.) with 3 µg total DNA per 35-mm dish. For imaging experiments, cells were kept in imaging medium (FluoroBrite DMEM supplemented with 10% FBS, 4 mM L-glutamine, and 25 mM HEPES).

Preparation of SNAP-alkene and reactivity evaluation

For in vitro experiments, His-tag was inserted into the C-terminus of SNAP-tag using Q5 site-directed mutagenesis kit (NEB) and pSNAP-tag(T7)-2 (NEB) as template to obtain pSNAP-C-His. BL21(DE3) cells harboring pSNAP-C-His were grown in LB broth to OD₆₀₀ 0.6–0.8, and then protein expression was induced with 1 mM IPTG and incubated for another 3 h. SNAP-tag protein was purified by Ni-NTA chromatography and protein yield was determined using Pierce BCA protein assay kit (Thermo Fisher). The strained alkenes were introduced into SNAP-tag by incubating 5 µM protein with 10 µM BG-alkene substrate in PBS with 1 mM DTT at 37 °C for 30 min. The DpTz-FL (1 mM stock in DMSO) reagent was then added to the reaction mixture to a final concentration of 100 µM, and the mixture was incubated at room temperature for 10 min. For in-gel fluorescence analysis, the reaction was quenched by boiling the sample with an equal volume of sample loading buffer. After SDS-PAGE, the protein gel was exposed to UV light on a transilluminator and photographed using a digital camera. For determination of reaction yield, the excess reagents were removed through buffer exchange with PBS using an Amicon Ultra 0.5 mL filter (EMD Millipore) and the protein mixture was analyzed by ESI-LC/MS. The reaction yield was calculated based on ion counts using the following equation: % conversion = $I_{\text{Product}} / (I_{\text{SNAP-alkene}} + I_{\text{Product}}) \times 100\%$.

Evaluation of SNAP-alkene reactivity in live cells

For live cell experiments, SNAP-tag gene was custom synthesized and inserted into the pCMV6-GLP-1R-myc-DDK (OriGene) vector after the signal sequence to obtain pCMV6-SNAP-GLP-1R. HEK293T cells expressing SNAP-GLP-1R were incubated with 5 µM BG substrate in 1 mL growth medium at 37 °C/5% CO₂ for 30 min. Cells were washed once

with growth medium then treated with 1 or 5 μM DpTz-AF647 or 5 μM DpTz-FL (diluted from a 1 mM stock solution in DMSO) with 1 mL growth medium at 37 °C/5% CO_2 for 30 min or 1 h. The cells were washed twice and incubated in 1 mL growth medium for 2 h before changing to imaging medium.

UAA incorporation in HEK293T cells

HEK293T cells were co-transfected with 4:1 ratio of pCMV-*Mm*PylRS-U6-tRNA (BocK, SphK) or pCMV-TCOKRS-U6-tRNA (TCOK) and the plasmid encoding GPCR bearing an amber codon at a pre-specified location. One hundred mM solution of UAA in DMSO was diluted in growth medium to a final concentration of 1 mM (SphK, TCOK) or 2 mM (BocK) and filtered through 0.2 μM PES then added to the cells followed by the transfection mixture. Cells were incubated for an additional 24–48 h then imaged directly.

Labeling of UAA-encoded GCGR and GLP-1R on live cell surface

A fresh solution of DpTz-AF647 or DpTz-Cy3 was prepared from a 1 mM stock in DMSO to a final concentration of 5 μM in 1 mL growth medium and filtered through 0.2 μM polyethersulfone membrane. The solution was added to HEK293T cells expressing BocK-, SphK-, or TCOK-encoded GCGR or SphK-encoded GLP-1R and incubated at 37 °C, 5% CO_2 , for 1 h. The cells were washed twice and incubated in 1 mL growth medium for 2 h before changing to imaging medium for laser scanning confocal microscopy.

cAMP accumulation assay

To characterize biological activity of wild type and mutant glucagon and GLP-1 receptors, glucagon- and GLP-1-stimulated cAMP responses in transiently transfected HEK293T cells were examined. Cell transfection, unnatural amino acid incorporation and Cy3 dye labeling were performed as described above. Forty-eight hours post-transfection, cells were lifted using non-enzymatic dissociation solution, washed sequentially with PBS and cAMP stimulation medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.01% soybean trypsin inhibitor, 0.2% bovine serum albumin, 0.1% bacitracin, and 1 mM 3-isobutyl-1-methylxanthine. Cells were distributed to a 96-well tissue culture plate at 50,000 cells per well and were stimulated with increasing concentrations (0 to 1 μM) of glucagon or GLP-1 at 37 °C for 30 min. After incubation, the medium was removed by gentle aspiration and cells were lysed with ice-cold 6% perchloric acid for 15 min with vigorous shaking. The cell lysates were adjusted to pH 6.0 with 30% KHCO_3 and assayed for cAMP levels in a 384-well white Optiplates using a LANCE kit from PerkinElmer (Waltham, MA) following the manufacturer's instructions. The assay was performed in duplicates and repeated in three independent experiments.

FACS analysis

For receptor expression measurement, HEK293T cells expressing GCGR-H372SphK ($\sim 10^6$ cells) were incubated with anti-GCGR (Alomone Labs; 1:50 dilution) in 100 μL FACS buffer (1 $\times$ DPBS, 1% BSA, 0.03% NaN_3) at room temperature for 1 h. The cells were washed three times with 250 μL FACS buffer each followed by incubation with allophycocyanin (APC)-conjugated anti-rabbit IgG(Ab')₂ fragment (Thermo Fisher; 1:150

dilution) in 100 μ L FACS buffer at room temperature for 1 h in the dark. The cells were washed three times, re-suspended in 1 mL FACS buffer, and analyzed using LSRFortessa Cell Analyzer (BD Biosciences). APC fluorescence was normalized to the wild-type GCGR and cell surface expression is depicted as % of wild type. To determine labeling yield, HEK293T cells expressing GCGR-H372SphK ($\sim 10^6$ cells) were labeled with DpTz-Cy3 followed by FACS analysis. The Cy3 fluorescence was normalized to that of SNAP-GCGR labeled with BG-Cy3.

Fluorescence microscopy

For quantification of UAA incorporation efficiency, the fluorescence of GFP, which was fused at the C-terminus of GCGR or GLP-1R, was measured using Lionheart FX automated live cell imager (BioTek Instruments) equipped with a 4 $\times$ /0.13 objective and a GFP filter (469/525). Fluorescence images of selected areas containing $\sim 10,000$ cells were acquired and processed using Gen5 software (BioTek Instruments), and the fluorescence of each mutant grown in the presence or absence of BocK were normalized to the wild-type GCGR-GFP or GLP-1R-GFP and expressed as % wild-type from two independent experiments. Confocal imaging was performed using a Zeiss LSM 710 equipped with PlanApochromat 20 $\times$ /0.8 M27 or 40 $\times$ /1.3 Oil DIC M27 objective. The excitation and emission profiles for each fluorophore are as follows: GFP (Ex 488/Em 493-598), FITC (Ex 490/Em 495-635), Cy3 (Ex 550/Em 555-680), Cy5 (Ex 640/Em 645-759), AlexaFluor 647 (Ex 635/Em 640-759). For dual color imaging, the profiles for each pair are as follows: GFP (Ex 488/Em 493-598)/AlexaFluor 647 (Ex 635/Em 640-759); Cy3 (Ex 535/Em 555-680)/Cy5 (Ex 640/Em 645-759).

GPCR internalization assay

HEK293T cells expressing the Cy3-labeled GCGR-SphK mutants were treated with 1 μ M GCG or GCG-Cy5 in growth medium at 37 $^{\circ}$ C, 5% CO₂ for 10 min. The medium containing the unbound ligand was switched to the imaging medium. For time-course experiments, cells were stimulated with 10 μ M GCG, and at each time point (5, 15, 30, 60 min) the unbound ligand was removed. The medium was switched to the imaging medium for immediate micrograph acquisition.

Time-lapsed imaging

HEK293T cells displaying the Cy3-labeled GCGR-H372 mutant were incubated in the imaging medium (DMEM, high glucose, 25 mM HEPES) at 37 $^{\circ}$ C, 5% CO₂ for 30 min. Cells were then placed on a Zeiss Axio Observer Fluorescence Microscope equipped with a 64 $\times$ objective and a Cy3 filter set (ex 538-562, em 570-640) and treated with 10 μ M GCG at 37 $^{\circ}$ C. The time-lapsed fluorescent images were acquired every 4 min for 38 min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the NIH (GM085092) for financial support, and Mr. Alan Siegel at SUNY Buffalo North Campus Imaging Facility for assistance with confocal microscopy.

References

1. Pocai A, Carrington PE, Adams JR, Wright M, Eiermann G, Zhu L, Du X, Petrov A, Lassman ME, Jiang G, Liu F, Miller C, Tota LM, Zhou G, Zhang X, Sountis MM, Santoprete A, Capito' E, Chicchi GG, Thornberry N, Bianchi E, Pessi A, Marsh DJ, SinhaRoy R. Diabetes. 2009; 58:2258–2266. [PubMed: 19602537]
2. Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, Findeisen H, Bruemmer D, Drucker DJ, Chaudhary N, Holland J, Hembree J, Abplanalp W, Grant E, Ruehl J, Wilson H, Kirchner H, Lockie SH, Hofmann S, Woods SC, Nogueiras R, Pfluger PT, Perez-Tilve D, DiMarchi R, Tschoop MH. Nat Chem Biol. 2009; 5:749–757. [PubMed: 19597507]
3. Siu FY, He M, de Graaf C, Han GW, Yang D, Zhang Z, Zhou C, Xu Q, Wacker D, Joseph JS, Liu W, Lau J, Cherezov V, Katritch V, Wang MW, Stevens RC. Nature. 2013; 499:444–449. [PubMed: 23863937]
4. Hollenstein K, Kean J, Bortolato A, Cheng RY, Dore AS, Jazayeri A, Cooke RM, Weir M, Marshall FH. Nature. 2013; 499:438–443. [PubMed: 23863939]
5. Katritch V, Cherezov V, Stevens RC. Annu Rev Pharmacol Toxicol. 2013; 53:531–556. [PubMed: 23140243]
6. Rasmussen SGF, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VRP, Sanishvili R, Fischetti RF, Schertler GFX, Weis WI, Kobilka BK. Nature. 2007; 450:383–387. [PubMed: 17952055]
7. Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SGF, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK. Science. 2007; 318:1266–1273. [PubMed: 17962519]
8. Harikumar KG, Lam PCH, Dong M, Sexton PM, Abagyan R, Miller LJ. J Biol Chem. 2007; 282:32834–32843. [PubMed: 17827151]
9. Dong M, Xu X, Ball AM, Makhoul JA, Lam PCH, Pinon DI, Orry A, Sexton PM, Abagyan R, Miller LJ. FASEB J. 2012; 26:5092–5105. [PubMed: 22964305]
10. Miller LJ, Chen Q, Lam PCH, Pinon DI, Sexton PM, Abagyan R, Dong M. J Biol Chem. 2011; 286:15895–15907. [PubMed: 21454562]
11. Xiang Z, Ren H, Hu YS, Coin I, Wei J, Cang H, Wang L. Nat Meth. 2013; 10:885–888.
12. Koole C, Reynolds CA, Mobarec JC, Hick C, Sexton PM, Sakmar TP. J Biol Chem. 2017; 292:7131–7144. [PubMed: 28283573]
13. Coin I, Katritch V, Sun T, Xiang Z, Siu Fai Y, Beyermann M, Stevens Raymond C, Wang L. Cell. 2013; 155:1258–1269. [PubMed: 24290358]
14. Rashidian M, Dozier JK, Distefano MD. Bioconjug Chem. 2013; 24:1277–1294. [PubMed: 23837885]
15. Ramil CP, An P, Yu Z, Lin Q. J Am Chem Soc. 2016; 138:5499–5502. [PubMed: 27082895]
16. Chatterjee A, Guo J, Lee HS, Schultz PG. J Am Chem Soc. 2013; 135:12540–12543. [PubMed: 23924161]
17. Peng T, Hang HC. J Am Chem Soc. 2016; 138:14423–14433. [PubMed: 27768298]
18. Lavis LD, Raines RT. ACS Chem Biol. 2008; 3:142–155. [PubMed: 18355003]
19. Ramil CP, Lin Q. Chem Commun. 2013; 49:11007–11022.
20. Lang K, Chin JW. Chem Rev. 2014; 114:4764–4806. [PubMed: 24655057]
21. Tian H, Naganathan S, Kazmi MA, Schwartz TW, Sakmar TP, Huber T. ChemBioChem. 2014; 15:1820–1829. [PubMed: 25045132]
22. Naganathan S, Ray-Saha S, Park M, Tian H, Sakmar TP, Huber T. Biochemistry. 2015; 54:776–786. [PubMed: 25524496]
23. Park M, Sivertsen BB, Els-Heindl S, Huber T, Holst B, Beck-Sickinger AG, Schwartz TW, Sakmar TP. Chem Biol. 2015; 22:1431–1436. [PubMed: 26548612]

24. Yu Z, Lin Q. *J Am Chem Soc.* 2014; 136:4153–4156. [PubMed: 24592808]
25. Kamber DN, Nazarova LA, Liang Y, Lopez SA, Patterson DM, Shih HW, Houk KN, Prescher JA. *J Am Chem Soc.* 2013; 135:13680–13683. [PubMed: 24000889]
26. Darko A, Wallace S, Dmitrenko O, Machovina MM, Mehl RA, Chin JW, Fox JM. *Chem Sci.* 2014; 5:3770–3776. [PubMed: 26113970]
27. Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J, Robillard MS. *Bioconjugate Chem.* 2013; 24:1210–1217.
28. Thalhammer F, Wallfaher U, Sauer J. *Tetrahedron Lett.* 1990; 31:6851–6854.
29. Yang J, Šekut J, Cole CM, Devaraj NK. *Angew Chem Intl Ed.* 2012; 51:7476–7479.
30. Blackman ML, Royzen M, Fox JM. *J Am Chem Soc.* 2008; 130:13518–13519. [PubMed: 18798613]
31. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K. *Nat Biotech.* 2003; 21:86–89.
32. Patterson DM, Nazarova LA, Xie B, Kamber DN, Prescher JA. *J Am Chem Soc.* 2012; 134:18638–18643. [PubMed: 23072583]
33. Mukai T, Kobayashi T, Hino N, Yanagisawa T, Sakamoto K, Yokoyama S. *Biochemical and Biophysical Research Communications.* 2008; 371:818–822. [PubMed: 18471995]
34. Pavlos NJ, Friedman PA. *Trends Endocrinol Metab.* 28:213–226.
35. Roed SN, Nøhr AC, Wismann P, Iversen H, Bräuner-Osborne H, Knudsen SM, Waldhoer M. *J Biol Chem.* 2015; 290:1233–1243. [PubMed: 25451942]
36. Kozma E, Demeter O, Kele P. *ChemBioChem.* 2017; 18:486–501. [PubMed: 28070925]
37. Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A. *BMC Genomics.* 2011; 12:14. [PubMed: 21214938]
38. Koole C, Wootten D, Simms J, Miller LJ, Christopoulos A, Sexton PM. *J Biol Chem.* 2012; 287:3642–3658. [PubMed: 22147710]
39. Dong M, Koole C, Wootten D, Sexton PM, Miller LJ. *Br J Pharmacol.* 2014; 171:1085–1101. [PubMed: 23889342]
40. Pott M, Schmidt MJ, Summerer D. *ACS Chem Biol.* 2014; 9:2815–2822. [PubMed: 25299570]
41. Schmied WH, Elsässer SJ, Uttamapinant C, Chin JW. *J Am Chem Soc.* 2014; 136:15577–15583. [PubMed: 25350841]
42. Niki I, Estrada Girona G, Kang JH, Paci G, Mikhaleva S, Koehler C, Shymanska NV, Ventura Santos C, Spitz D, Lemke EA. *Angew Chem Intl Ed.* 2016; 55:16172–16176.
43. Zheng Y, Lewis TL Jr, Igo P, Polleux F, Chatterjee A. *ACS Synth Biol.* 2017; 6:13–18. [PubMed: 27482719]
44. Rahim MK, Kota R, Haun JB. *Bioconjug Chem.* 2015; 26:352–360. [PubMed: 25584926]
45. Zhang H, Qiao A, Yang D, Yang L, Dai A, de Graaf C, Reedtz-Runge S, Dharmarajan V, Zhang H, Han GW, Grant TD, Sierra RG, Weierstall U, Nelson G, Liu W, Wu Y, Ma L, Cai X, Lin G, Wu X, Geng Z, Dong Y, Song G, Griffin PR, Lau J, Cherezov V, Yang H, Hanson MA, Stevens RC, Zhao Q, Jiang H, Wang MW, Wu B. *Nature.* 2017; 546:259–264. [PubMed: 28514451]
46. Zhang Y, Sun B, Feng D, Hu H, Chu M, Qu Q, Tarrasch JT, Li S, Sun Kobilka T, Kobilka BK, Skiniotis G. *Nature.* 2017; 546:248–253. [PubMed: 28538729]
47. Jazayeri A, Rappas M, Brown AJH, Kean J, Errey JC, Robertson NJ, Fiez-Vandal C, Andrews SP, Congreve M, Bortolato A, Mason JS, Baig AH, Teobald I, Doré AS, Weir M, Cooke RM, Marshall FH. *Nature.* 2017; 546:254–258. [PubMed: 28562585]

Figure 1.

Comparison of spirohexene reactivity with those of *trans*-cyclooctene (TCO, single axial diastereomer) and 1,3-dialkyl substituted cyclopropene (Cyp) toward DpTz in the tetrazine ligation in vitro and on live cell surface. (a) Scheme for introduction of strained alkenes into SNAP protein and subsequent labeling with DpTz-FL. (b) In-gel fluorescence analysis of SNAP labeling via tetrazine ligation. The SNAP protein (5 μ M) was incubated with 10 μ M of BG compound at 37 $^{\circ}$ C for 1 h, and then with 100 μ M of DpTz-FL at room temperature for 10 min (see experimental section for details). Structures of the BG compounds are shown on the top. Equal amount of protein loading was verified by Coomassie staining of the same gel. (c) LCQ-MS analysis of the product mixture after tetrazine ligation with DpTz-FL. (d) Scheme for introduction of the strained alkenes into SNAP-tag of the SNAP-GLP-1R fusion protein and subsequent reaction with DpTz-FL in live HEK293T cells. (e) Confocal

micrographs of the alkene-encoded SNAP-GLP-1R proteins after labeling with DpTz-FL.
Scale bar = 20 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

Effects of unnatural amino acid mutagenesis on GCGR expression and ligand binding. (a) Relative expressions of the GCGR-GFP UAA mutants in the presence or absence of BocK based on GFP fluorescence. For quantification, expression level of the wild-type GCGR-GFP was set at 100%. (b) The cAMP response of the GCGR-GFP mutants to glucagon stimulation. For quantification, maximum cAMP response of the wild-type GCGR was set at 100%. (c) The Cy5-labeled glucagon peptide (GCG-Cy5, 1 μ M) induced internalization of the WT and SphK-encoded GCGR mutants. Representative internalized GCGR receptors in complex with the GCG-Cy5 ligand are marked with white arrows in Cy5 channel. EGFP was co-expressed both as a transfection control and as a marker of cytoplasm in this experiment. Scale bar = 20 μ m.

Figure 3.

Bioorthogonal labeling of the strained alkene-encoded GCGR mutants in live HEK293T cells and characterization of their binding to a fluorescent glucagon peptide ligand. (a) Evaluation of labeling efficiency and specificity by the tetrazine ligation with DpTz-AF647 in HEK293T cells. The GCGR-GFP mutants encoding BocK, TCOK, and SphK at position E55 or D370 were used in this analysis. See Figure S8-S10 in SI for mutations at other positions. (b) Residues on the extracellular loop 3 (ECL3) of GCGR (PDB: 4L6R), showing their relative position with respect to the exterior surface of cytoplasmic membrane. (c) Measurement of cell surface expression of GCGR and its mutants by FACS using rabbit anti-GCGR and anti-rabbit-APC antibodies. The expression of wild-type GCGR was set at 100%. Determination of the Sph-DpTz ligation yield by FACS. For quantification, the BG-Cy3 labeled SNAP-GCGR was set as 100%.

Figure 4.

Functional characterization of the Cy3-labeled GCGR-H372SphK mutant. (a) Binding of 1 μM GCG-Cy5 ligand and subsequent internalization of the activated Cy3-labeled GCGR mutants. Scale bar = 20 μm . (b) The cAMP responses of wild-type GCGR, GCGR-H372SphK, GCGR-H372Cy3, and GCGR-H372TAG mutants in HEK293T cells to glucagon stimulation. (c) Internalization time course of the Cy3-labeled GCGR-H372SphK after stimulation with 10 μM glucagon for 5, 15, 30, and 60 minutes. Scale bar = 20 μm .

Figure 5.

Unnatural amino acid mutagenesis and bioorthogonal labeling of GLP-1R in live cells. (a) Relative expression levels of the BocK-encoded GLP-1R-GFP mutants. (b) The cAMP responses of the BocK-encoded GLP-1R-GFP mutants. (c) Labeling of the SphK-encoded GLP-1R mutants by DpTz-FL via tetrazine ligation in live HEK293T cells. Scale bar = 20 μ m. (d) GLP-1-induced internalization of the Cy3-labeled GLP-1R-T378SphK in HEK293T cells. Cells were stimulated with 1 μ M GLP-1 for 15 minutes before image acquisition. Scale bar = 20 μ m. (e) The cAMP response of the wild-type GLP-1R, GLP-1R-T378SphK, GLP-1R-T378Cy3, and GLP-1R-T378TAG mutants to GLP-1 stimulation.

Reaction scheme:

Strained alkenes:

Tetrazine reagents:

Chart 1.

Table 1

Effect of unnatural amino acid mutagenesis of GCGR and its bioorthogonal labeling on glucagon-induced intracellular cAMP accumulation. pEC_{50} value represents negative logarithm of the concentration of glucagon that produces half of the maximal response, E_{max} . E_{max} is presented as % of the WT response. All values are expressed as mean $\pm$ S.E.M. of three individual experiments performed in duplicate.

GCGR variant	pEC_{50}	E_{max}
WT	10.7 ± 0.1	101 ± 3
H372SphK	9.6 ± 0.2	88 ± 4
H372SphK-Cy3	9.7 ± 0.4	83 ± 8
H372TAG	8.7 ± 0.2	35 ± 2
H372TAG (no RS)	9.9 ± 0.3	12 ± 1

Table 2

Effect of unnatural amino acid mutagenesis of GLP-1R and its bioorthogonal labeling on GLP-1-induced cAMP accumulation. pEC_{50} values represent negative logarithm of the concentration of agonist that produces half of the maximal response, E_{max} . E_{max} is presented as a % of the WT response. All values are expressed as mean $\pm$ S.E.M. of three individual experiments performed in duplicate.

GLP-1R variant	pEC_{50}	E_{max}
WT	10.7 ± 0.1	102 ± 2
T378SphK	10.3 ± 0.1	98 ± 2
T378SphK-Cy3	10.4 ± 0.2	90 ± 3
T378TAG	9.0 ± 0.1	33 ± 2