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Title: Differential Impact of Amino Acid Substitutions on Critical Residues of the Human Glucagon-Like Peptide-1 Receptor (GLP-1R) Involved in Peptide Activity and Small Molecule Allostery.

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Running title: GLP-1R residues important for activity and modulation

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Abbreviations: BETP, 4-(3-benzyloxy)phenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; CHO, Chinese hamster ovary; compound 2, 6,7-dichloro-2-methylsulfonyl-3-*tert*-butylaminoquinoxaline; CRF, corticotropin releasing factor; DM, diabetes mellitus; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein-coupled receptor; ICL, intracellular loop; PAM, positive allosteric modulator; pERK1/2, phosphorylated extracellular signal regulated kinase 1 and 2; PFA, paraformaldehyde; SNP, single nucleotide polymorphism; TM, transmembrane.

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Abstract

The glucagon-like peptide-1 receptor (GLP-1R) is a Class B G protein-coupled receptor (GPCR) that has a critical role in the regulation of glucose homeostasis, principally though regulation of insulin secretion. The receptor system is highly complex, with the ability to be activated by both endogenous (GLP-1(1-36)NH_2, GLP-1(1-37), GLP-1(7-36)NH_2, GLP-1(7-37), oxyntomodulin) and exogenous (exendin-4) peptides in addition to small molecule allosteric agonists (6,7-dichloro-2-methylsulfonyl-3-*tert*-butylaminoquinoxaline (compound 2), 4-(3-benzyloxy)phenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine (BETP)). Furthermore, the GLP-1R is subject to single nucleotide polymorphic variance, resulting in amino acid changes in the receptor protein. In this study, we investigated two polymorphic variants that have previously been reported to impact peptide-mediated receptor activity (M149) and small molecule allostery (C333). These residues were mutated to a series of alternate amino acids and their functionality monitored across physiologically significant signaling pathways including cAMP, extracellular signal-regulated kinase 1 and 2 phosphorylation (pERK1/2) and intracellular Ca^{2+} mobilization, in addition to peptide binding and cell surface expression. We observed that residue 149 is highly sensitive to mutation, with almost all peptide responses significantly attenuated at mutated receptors. However, most reductions in activity were able to be restored by the small molecule allosteric agonist, compound 2. Conversely, mutation of residue 333 has little impact on peptide-mediated receptor activation, but this activity is unable to be modulated by compound 2 to the same extent as that observed at the wildtype receptor. These results provide insight into the

importance of residues 149 and 333 in peptide function and highlight the complexities of allosteric modulation within this receptor system.

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Introduction

The glucagon-like peptide 1 receptor (GLP-1R) is a Class B peptide hormone G protein-coupled receptor (GPCR) with physiologically important actions including increases in insulin biosynthesis and secretion from pancreatic β-cells and decreases in β-cell apoptosis, gastric emptying and peripheral tissue resistance to insulin. For these reasons, the GLP-1R is one of the key targets in the development of therapeutics for type II diabetes mellitus (DM). However, with an increasing interest in establishing novel, long acting and orally available therapeutics that eliminate or at least significantly reduce detrimental side effects, the pharmacological complexities of targeting this receptor system are becoming evident.

The most well documented consequence of GLP-1R activation is enhanced cAMP production, which along with cell membrane depolarization and the influx of Ca^{2+}, is critical in the biosynthesis and exocytosis of insulin from pancreatic β-cells (Fehmann and Habener, 1992; Lu et al., 1993). However, the GLP-1R can couple via other G protein-dependent mechanisms, including $G\alpha_i$, $G\alpha_o$ and $G\alpha_{q/11}$ (Hallbrink et al., 2001; Montrose-Rafizadeh et al., 1999), as well as via β-arrestin recruitment and signaling (Jorgensen et al., 2007; Jorgensen et al., 2005; Sonoda et al., 2008). Furthermore, with the ability to be activated by multiple endogenous agonists (GLP-1(1-36)NH_2, GLP-1(1-37), GLP-1(7-36)NH_2, GLP-1(7-37) and oxyntomodulin) as well as the exogenous peptide agonist exendin-4 (Byetta®) that is currently used as a type II diabetic treatment and allosteric ligands such as compound 2, the phenomenon of biased agonism can clearly be observed at this receptor (Kenakin, 1995, 2011; Koole et al., 2012a; Koole et al., 2012b; Koole et al., 2010). Adding to the complexity of this receptor system, recent

pharmacological analysis of GLP-1R single nucleotide polymorphisms (SNPs) (Beinborn et al., 2005; Fortin et al., 2010; Koole et al., 2011) identified two variants that significantly influence receptor function; M149 (transmembrane domain (TM) 1), which attenuates endogenous and exogenous peptide-mediated receptor function (Beinborn et al., 2005; Koole et al., 2011), and C333 (intracellular loop (ICL) 3), which reduces the allosteric agonism of the GLP-1R small molecule compound 2, as well as significantly impacting its modulatory profile (Koole et al., 2011). While population analysis of these receptor variants suggest a low heterozygous frequency and unknown homozygous frequency, for at least the M149 variant, there has been a direct implication in the onset of type II DM (Tokuyama et al., 2004). Moreover, the significant loss of peptide function at this receptor variant would also suggest that subjects administered peptide mimetics such as exendin-4 would experience limited effectiveness in management of the condition (Koole et al., 2011).

In the absence of high-resolution crystal structures of the GLP-1R in its entirety, and in fact any Class B GPCR as a whole entity, the structural role of these residues and their influence on the mechanistic function of the receptor is largely unclear. However, with the emergence of two Class B TM crystal structures (Hollenstein et al., 2013; Siu et al., 2013) and a plethora of mutagenesis (Coopman et al., 2011; Koole et al., 2012a; Koole et al., 2012b; Lopez de Maturana and Donnelly, 2002; Lopez de Maturana et al., 2004; Wootten et al., 2013) and photoaffinity labeling data (Al-Sabah and Donnelly, 2003; Chen et al., 2009, 2010; Coin et al., 2013; Dong et al., 2007; Dong et al., 2011; Dong et al., 2004; Miller et al., 2011), structurally and functionally important components of the GLP-1R can begin to be predicted and complementary molecular models can be further

refined. In this study, we have created a series of mutations at two GLP-1R residues subject to polymorphic variance (amino acids 149 and 333; Fig 1) at which receptor function is significantly affected, to more broadly examine their involvement in receptor structure and function. We have observed that mutation of residue 149, at which Thr most frequently occurs, is poorly tolerated in the context of peptide-mediated receptor activation but not that of the allosteric agonist compound 2. In addition, mutants of this residue with significantly reduced peptide activity were able to have at least partial restoration of function through compound 2-mediated allosteric modulation of the receptor. Conversely, at residue 333, at which Ser most frequently occurs, there was little influence of receptor mutants on peptide function in any detected output, however, modulation of oxyntomodulin-induced cAMP formation by compound 2 was significantly attenuated despite compound 2 retaining agonism. Together, these results not only enhance our understanding of the role of SNPs in receptor activity, but also facilitate the refinement of models in understanding the complex molecular mechanisms involved in GLP-1R activity.

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Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), hygromycin-B and Fluo-4 acetoxymethyl ester were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). The QuikChange™ site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). AlphaScreen™ reagents, Bolton-Hunter reagent (^{125}I) and 384-well ProxiPlates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). SureFire™ ERK1/2 reagents were generously supplied by TGR Biosciences (Adelaide, SA, Australia). Sigma*Fast o*-phenylenediamine dihydrochloride tablets and antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compound 2 was generated according to a method published previously (Teng et al., 2007) to a purity of >95%, and compound integrity was confirmed by NMR. GLP-1 and GLP-1 peptide analogs were purchased from American Peptide (Sunnyvale, CA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade.

Receptor Mutagenesis. SNPs of the GLP-1R with pharmacological profiles deviating from 'wildtype', as determined from our previous study (Koole et al., 2011), were mutated to a selection of amino acids. Mutations were introduced to an N-terminally double c-myc labeled wildtype human GLP-1R in the pEF5/FRT/V5-DEST destination vector (Invitrogen); this receptor had equivalent pharmacology to the untagged human GLP-1R (data not shown). Mutagenesis was carried out using oligonucleotides for site-directed mutagenesis from GeneWorks (HindMarsh, SA, Australia) (Supplementary Table S1) and the QuikChange™ site-directed mutagenesis kit (Stratagene). Sequences

of receptor clones were confirmed by cycle sequencing as previously described (May et al., 2007). In this study, wildtype GLP-1R is comprised of T149 and S333.

Transfections and Cell Culture. Wildtype and mutant human GLP-1R were isogenically integrated into FlpIn-Chinese hamster ovary (FlpInCHO) cells (Invitrogen) and selection of receptor-expressing cells accomplished by treatment with 600 μg ml^{-1} hygromycin-B as previously described (May et al., 2007). Transfected and parental FlpInCHO cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37°C in 5% CO_2.

Radioligand Binding Assay. FlpInCHO wildtype and mutant human GLP-1R cells were seeded at a density of 3 x 10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and radioligand binding carried out at 4°C as previously described (Koole et al., 2011). For each cell line in all experiments, total binding was defined by 0.5 nM ^{125}I-exendin(9-39) alone, and nonspecific binding was defined by 1 μM exendin(9-39). For analysis, data are normalized to the B_0 value for each individual experiment. Of note, the condition of assay for radioligand binding and functional experiments are different and as such cannot be directly compared.

cAMP Accumulation Assay. FlpInCHO wildtype and mutant human GLP-1R cells were seeded at a density of 3 x 10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and cAMP detection carried out as previously described (Koole et al., 2010). For interaction studies, increasing concentrations of peptide and 3 μM compound 2 were added simultaneously, and cAMP accumulation measured after 30 min of cell stimulation. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently

normalized to the response of 100 μM forskolin in each cell line. Agonist stimulation and interaction studies were performed as two different series of experiments, on different cell passages.

pERK1/2 Assay. FlpInCHO wildtype and mutant human GLP-1R cells were seeded at a density of 3 x 10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2. Receptor-mediated pERK1/2 was determined using the AlphaScreen™ ERK1/2 SureFire™ protocol as previously described (May et al., 2007). Initial pERK1/2 time course experiments were performed over 1 h to determine the time at which agonist-mediated pERK1/2 was maximal. Subsequent experiments were then performed at the time required to generate a maximal pERK1/2 response (6 min). Data were normalized to the maximal response elicited by 10% FBS in each cell line, determined at 6 min (peak FBS response).

$_iCa^{2+}$ Mobilization Assay. FlpInCHO wildtype and mutant human GLP-1R cells were seeded at a density of 3 x 10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2, and receptor-mediated $_iCa^{2+}$ mobilization determined as previously described (Werry et al., 2005). Fluorescence was determined immediately after peptide addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings taken every 1.36 s for 120 s. Peak magnitude was calculated using five-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP.

Cell Surface Receptor Expression. FlpInCHO wildtype and mutant human GLP-1R cells, with receptor DNA previously incorporated with an N-terminal double c-myc

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epitope label, were seeded at a density of 25 x 10^4 cells/well into 24-well culture plates and incubated overnight at 37°C in 5% CO_2, washed three times in 1 x PBS and fixed with 3.7% paraformaldehyde (PFA) at 4°C for 15 min. Cell surface receptor detection was then performed as previously described (Koole et al., 2011). Data were normalized to the basal fluorescence detected in FlpInCHO parental cells. Specific ^{125}I-exendin(9-39) binding at each receptor mutant, as identification of functional receptors at the cell surface, was also determined (corrected for nonspecific binding using 1 μM exendin(9-39)).

Data Analysis. All data were analyzed using Prism 5.04 (GraphPad Software Inc., San Diego, CA, USA). For all analyses the data were unweighted and each *y* value (mean of replicates for each individual experiment) was considered an individual point. Concentration response signaling data were analyzed using a three-parameter logistic equation as previously described (May et al., 2007):

$$E=\text{Bottom}+\frac{(\text{Top-Bottom})[A]}{[A]+[EC_{50}]} \quad (1)$$

where *Bottom* represents the *E* value in the absence of ligand(s), *Top* represents the maximal stimulation in the presence of ligand(s), [*A*] is the molar concentration of ligand, and EC_{50} represents the molar concentration of ligand required to generate a response halfway between *Top* and *Bottom*. Similarly, this equation was used in the analysis of inhibition binding data, instead replacing EC_{50} with IC_{50}. In this case, *Bottom* defines the specific binding of the radioligand that is equivalent to non-specific ligand binding, whereas *Top* defines radioligand binding in the absence of a competing ligand, and the IC_{50} value represents the molar concentration of ligand required to generate a response

halfway between *Top* and *Bottom*. IC_{50} values obtained were then corrected for radioligand occupancy as previously described (Cheng and Prusoff, 1973) using the radioligand affinity (K_i) experimentally determined for each mutant.

To quantify efficacy in the system, all data were fitted with an operational model of agonism (Black and Leff, 1983):

$$E=\text{Bottom}+\frac{(\text{Top-Bottom})\tau[A]}{\tau[A]+[A]+K_A} \qquad (2)$$

where *Top* represents the maximal stimulation in the system; K_A is the agonist-receptor dissociation constant, in molar concentration; τ is the estimated measure of efficacy in the system, which incorporates both signaling efficacy and receptor density; and all other parameters are as defined for Equation 1. Constraints for this model were determined by fitting the most efficacious peptide with the following equation:

$$E=\text{Bottom}+\frac{(E_m\text{-Bottom})[A]}{[A]+[EC_{50}]} \qquad (3)$$

The value obtained for the system maximum (E_m) was then globally constrained as the parameter, *Top*, in the operational model (Equation 2) when applied at each mutant receptor. All estimated τ values were then corrected to cell surface expression (τ_c) as determined by percent specific ^{125}I-exendin(9-39) binding, and errors propagated from both τ and cell surface expression relative to wildtype receptor. Of note, differences in functional K_A values derived from fitting the operational model may arise from the presence of non-interconverting states that are unique to ligand-receptor-effector complexes (Holst et al., 2001; Leff et al., 1997; McPherson et al., 2010; Nijmeijer et al.,

2012; Strachan et al., 2010; Zheng et al., 2008), and this is observed for peptide agonists at the wildtype GLP-1R (Supplementary Table S2).

Statistics. Changes in peptide affinity, potency, efficacy, and cell surface expression of human GLP-1R mutants in comparison to wildtype human GLP-1R control were statistically analyzed with one-way analysis of variance and Dunnett's post test, and significance accepted at $p < 0.05$.

Results

Mutation of residue 149 or 333 has minimal impact on functional human GLP-1R expression at the cell surface. Wildtype N-terminally c-myc tagged human GLP-1R or receptors incorporating the mutations of residues 149 or 333 were isogenically introduced into FlpInCHO host cells by recombination, and cell surface expression determined through antibody detection of the c-myc epitope label (Table 1). In this study, mutation of residue 333 to either Ala or Val caused little deviation in cell surface expression in comparison to the wildtype human GLP-1R. In contrast, mutation of residue 149 to Val or Tyr significantly reduced receptor cell surface expression, while mutation of residue 149 to Cys significantly increased cell surface expression in comparison to wildtype control. No significant change in specific ^{125}I-exendin(9-39) binding was observed, although the C149 mutant trended towards increased binding (p = 0.11) consistent with the cell surface expression as monitored by c-myc antibody binding (Table 1). In addition, there was no significant difference in the level of receptor expression as determined by ^{125}I-exendin(9-39) binding. Wildtype receptor expression was 1.87 ± 0.33 million receptors/cell, and the mean level of expression across all mutants was 1.55 ± 0.32 million receptors/cell (data not shown).

Mutation of residue 149 but not 333 of the human GLP-1R significantly affects peptide agonist binding affinity but not antagonist exendin(9-39) binding affinity. Binding affinity of orthosteric GLP-1R peptide ligands at each of the mutant receptors was determined through equilibrium binding in the presence of the radiolabeled antagonist, ^{125}I-exendin(9-39) (Fig 2, Table 1). Homologous competition identified no significant changes in antagonist exendin(9-39) binding affinity at any mutant receptor (Table 1).

Consistent with results previously published (Koole et al., 2012a), complete inhibition curves were unable to be determined in the presence of GLP-1(1-36)NH_2 at any receptor mutant, and given the small window for competition within the concentration range tested, any deviations in binding affinity of this peptide at receptor mutants in comparison to wildtype control were difficult to interpret (Fig 2A). For the remaining agonist peptides, no significant changes in binding affinity were observed for either Ala or Val substitutions at residue 333, while decreases in binding affinity of GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin were observed at all residue 149 receptor mutants (Fig 2B-D, Table 1). However, the extent of affinity reduction was variable depending on both the residue substituted as well as the peptide present, with an overall greater effect of mutation on the affinity of GLP-1(7-36)NH_2 than exendin-4 or oxyntomodulin (Table 1). This is clearly evident at mutant receptors A149 and V149, with fold reductions of 79 for exendin-4 and oxyntomodulin at both mutants, but 200 and 251, respectively, for GLP-1(7-36)NH_2. Similarly, mutation of residue 149 to Phe resulted in fold decreases in binding affinity of 158, 316 and 501 for exendin-4, oxyntomodulin and GLP-1(7-36)NH_2, respectively. Perhaps not surprisingly, mutation of residue 149 to Ser, which has properties of greatest similarity to that of the most frequently occurring residue Thr had the least effect on peptide binding affinity, albeit that most reductions still reached statistical significance.

Most mutations of residue 149 but not 333 of the human GLP-1R significantly decrease peptide-mediated cAMP accumulation. While the GLP-1R is recognized as a pleiotropically coupled receptor, it is most well characterized for its role in enhancing adenylate cyclase activity and promoting the formation of cAMP, which is directly linked

to the secretion of insulin (Baggio and Drucker, 2007). Consequently, we assessed the ability of each mutant receptor to augment cAMP accumulation in the presence of each peptide agonist (Fig 3, Table 2). Consistent with effects on binding affinity, no significant effect of mutation of residue 333 on peptide potency in cAMP accumulation was observed (Table 2). Taking into account cell surface receptor expression and pathway coupling efficiency, application of the operational model to yield the operational measure of efficacy (τ_c) illustrated a general trend for decreased peptide-mediated cAMP coupling at both Ala and Val mutants of residue 333, albeit that this did not reach statistical significance. In contrast, decreases in peptide potency in cAMP were observed for all mutant receptors of residue 149 (Fig 3, Table 2). In agreement with binding data, peptide-mediated cAMP was least affected at S149 with respect to wildtype (T149), and this was also reflected by no significant differences in coupling efficacy (τ_c) when the operational model was applied (Table 2). However, there were trends for decreases in coupling efficacy in the presence of GLP-1(7-36)NH_2 and oxyntomodulin but increases in the presence of GLP-1(1-36)NH_2 and exendin-4, suggesting that the additional methyl group of Thr may contribute to discrimination of orthosteric peptide signaling profiles. Mutation of residue 149 to Cys resulted in decreased peptide potency, however, this only reached statistical significance for oxyntomodulin. Analysis with the operational model revealed no significant deviations in pathway coupling efficacy (τ_c) at C149 in the presence of any peptide, although there was a trend toward decreases for all peptides in comparison to the wildtype (T149) receptor. Significant reductions in peptide-mediated cAMP were noted with mutation of T149 to Ala, Phe, Ile, Val and Tyr, with only weak responses detected and potency values unable to be determined for GLP-1(1-36)NH_2,

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GLP-1(7-36)NH_2 and oxyntomodulin. Despite the inability to determine peptide potency values at these mutants, the rank order of reduction in peptide-mediated cAMP was consistent for all peptides; Ala>Val>Phe>Ile>Tyr (Fig 3).

Most mutations of residue 149 but not 333 of the human GLP-1R abolish $_iCa^{2+}$ mobilization. Consistent with work presented previously (Koole et al., 2010), $_iCa^{2+}$ was weakly coupled to GLP-1R activation in FlpInCHO cells, with no notable response for GLP-1(1-36)NH_2 at either the wildtype (T149, S333) or any receptor mutant (data not shown). Similarly, complete concentration-response curves in the presence of oxyntomodulin could not be established over the concentration range tested in this study. Comparison of responses at the highest peptide concentration tested (1 μM) revealed that mutation of residue 333 caused small increases in oxyntomodulin-mediated $_iCa^{2+}$ mobilization, however this was not statistically significant (Table 3). Increases in E_{max} were also observed in GLP-1(7-36)NH_2- and exendin-4-mediated $_iCa^{2+}$ mobilization responses at both 333 mutants, despite little deviation in peptide potency. While this increase in E_{max} reached statistical significance for GLP-1(7-36)NH_2, these data did not translate into significantly enhanced pathway coupling efficacy (τ_c) as determined through operational modeling (Table 3). Almost all substitutions at residue 149 had a significant impact on peptide-mediated $_iCa^{2+}$ mobilization (Fig 4). The exception was S149 that displayed similar activity to that of the wildtype (T149) GLP-1R in the presence of all measurable peptide responses, reflected in the coupling efficacy (Fig 4, Table 3). Interestingly, C149 showed no significant changes in peptide potency for either GLP-1(7-36)NH_2 or exendin-4, but significant attenuation of maximal response for exendin-4 and oxyntomodulin (the latter a measure of response at 1 μM). This was also

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reflected in the estimated coupling efficacy (τ_c), whereby although both GLP-1(7-36)NH_2 and exendin-4 had reduced efficiency in the Ca^{2+} pathway, only exendin-4 reached statistical significance, indicating possible ligand specific effects of this residue on peptide activity (Table 3).

Most mutations of residue 149 but not 333 of the human GLP-1R significantly decrease peptide-mediated pERK1/2. Peptide-mediated pERK1/2 was measured at 6 min for each receptor mutant, the time at which maximal stimulation of pERK1/2 occurred at the wildtype GLP-1R (data not shown). For mutants with measurable peptide-mediated pERK1/2 responses, there was no significant alteration of peptide potency (Fig 5, Table 4). This is consistent with what we have previously seen at GLP-1R mutants, indicating that receptor mutation generically has a lesser impact on coupling to ERK1/2 signaling (Koole et al., 2011). In accord with $_{i}Ca^{2+}$ mobilization data, there were no significant alterations in peptide potency at either mutant of residue 333, yet both mutants displayed notable increases in E_{max} that were in turn reflected in an increase in pathway coupling efficacy. However, these increases did not reach statistical significance for any peptides (Table 4). Receptor mutants F149, I149, V149 and Y149 profoundly affected pERK1/2 signaling, with little to no detectable responses for both GLP-1 peptides, and significant effects on maximal responses for exendin-4 and oxyntomodulin (Fig 5, Table 4). However, in most cases operational modeling revealed no statistically significant changes in coupling efficiency (τ_c) at these mutants. Mutation of 149 to either Ala or Cys significantly impacted the E_{max} of both GLP-1(7-36)NH_2 and oxyntomodulin, but had little effect on exendin-4. However, application of the operational model revealed that while all peptides had reduced coupling efficacy (τ_c) at these mutants, none reached

statistical significance. As predicted, there was little deviation in peptide responses at the S149 mutant.

Effect of residue 149 or 333 of the human GLP-1R on the signaling profile of the allosteric agonist compound 2. Previously, we demonstrated that despite a loss of peptide agonist binding and signaling at the naturally occurring M149 receptor variant, the agonist profile of the small molecule GLP-1R allosteric ligand compound 2 was retained (Koole et al., 2011). In the present study, mutation of residue 333 did not significantly affect the potency of compound 2 in either cAMP or pERK1/2 signaling profiles (Table 2 and 4). Despite significant reductions observed at the polymorphic variant C333, A333 and V333 did not display any significant effects on compound 2 agonism. Mutation of 149 did not significantly affect the potency of compound 2 in either cAMP accumulation or pERK1/2 outputs (Table 2 and 4). Coupling efficacy in pERK1/2 increased for A149, F149, S149, and Y149, albeit that these increases were not statistically significant (Table 4). Consistent with previous studies, no measurable agonism was observed for compound 2 in $_{i}Ca^{2+}$ mobilization at either the wildtype or any of the mutant receptors (data not shown).

Effect of residue 149 or 333 of the human GLP-1R on the modulatory profile of compound 2. We have previously shown that the loss of agonist peptide binding and cAMP signaling at the naturally occurring M149 receptor variant could be partially rescued in the presence of compound 2 (Koole et al., 2011). In addition, the C333 receptor variant was not modulated by compound 2 to the extent observed at the wildtype GLP-1R (S333) (Koole et al., 2011). In the present study, we have therefore examined the role of compound 2 in modulating peptide-induced cAMP responses of residue 149

and 333 mutants. Similar to previous observations, there was significant positive modulation of oxyntomodulin-mediated cAMP with the addition of compound 2 at the wildtype GLP-1R, but no significant effects on GLP-1(1-36)NH_2, GLP-1(7-36)NH_2 or exendin-4 (Table 5, Supplementary Fig S1-4; Koole et al., 2010). While no modulation of these latter peptides was observed at either 333 mutants, compound 2 also failed to significantly modulate the oxyntomodulin responses at A333 and V333 (Table 5, Supplementary Fig S4). Similar to wildtype GLP-1R, mutation of residue 149 to Ser had little effect on the modulatory profile of compound 2, with a substantial enhancement of oxyntomodulin potency but no significant effect on other peptides (Table 5, Supplementary Fig S1-4). At all other substitutions of residue 149, significant compound 2-mediated augmentation of oxyntomodulin potency was observed, however the extent of modulation was somewhat variable amongst mutants; greatest detectable recovery of potency was observed at the A149 mutant while only modest recovery was seen at the F149 mutant (100- and 16-fold enhancement of oxyntomodulin potency, respectfully) (Table 5, Supplementary Fig S4). As seen with the M149 receptor variant (Koole et al., 2011), in addition to modulation of oxyntomodulin, compound 2 also positively modulated most other peptide responses of 149 mutant receptors (Table 5, Supplementary Fig S1-3). In several cases (F149, I149, V149, Y149), compound 2 restored function of undetectable or undefined cAMP responses (Table 5, Supplementary Fig S1-4). This was particularly evident for Y149, with no detectable cAMP response for low potency agonists GLP-1(1-36)NH_2 and oxyntomodulin, but recovery of a detectable peptide response in the presence of compound 2. Despite positively modulating most peptide responses at 149 mutants, the extent of modulation by compound 2 was not consistent

across all mutants. Clear examples of this are A149, for which compound 2 modulated GLP-1(7-36)NH_2 and oxyntomodulin potency to a greater extent than GLP-1(1-36)NH_2 and exendin-4 (fold changes of 63, 100, 6 and 6, respectively), while at F149, exendin-4 potency was modulated to a greater extent than oxyntomodulin (fold changes of 200 and 16, respectively) (Table 5). Interestingly, compound 2 modulated both GLP-1(7-36)NH_2 and oxyntomodulin at C149, but had very little influence on GLP-1(1-36)NH_2 and exendin-4 (fold changes of 8, 32, 1 and 3, respectively) (Table 5). These subtle changes in modulation profiles suggest an important role of residue 149 in directing probe dependent effects on cooperativity.

Discussion

Class B GPCRs are important regulators for a number of physiological processes. Consequently, they have become valuable therapeutic targets for multiple disorders including neurodegenerative and inflammatory conditions (vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide receptors) (Abad et al., 2006; Brenneman, 2007), bowel disorders (GLP-2 receptors) (Hornby and Moore, 2011), chronic stress (corticotropin releasing factor (CRF) receptors) (Gilligan and Li, 2004), bone-related disorders (calcitonin and parathyroid hormone receptors) (Mulder et al., 2006) and type II DM (glucagon, amylin and GLP-1Rs) (Adeghate and Kalasz, 2011; Brubaker, 2007; Estall and Drucker, 2006). Many of these GPCRs exhibit single nucleotide polymorphic variance, with the variant proteins linked to development of multiple diseases, and there is additional potential to impact the effectiveness of treatments targeted to that receptor (Hager et al., 1995; Sadee et al., 2001; Schipani et al., 1999; Siani et al., 2001; Taboulet et al., 1998; Tang and Insel, 2005).

The naturally occurring GLP-1R variant, M149, markedly reduces peptide-mediated functional responses *in vitro*, suggesting that this would engender a pathophysiological phenotype. In accord, possession of this variant has been associated with poor glycemic control (Tokuyama et al., 2004). The mechanistic basis behind this loss of function receptor variant is largely unclear due to the paucity of structural information within this class of GPCRs. Consequently, we have substituted amino acids with different physicochemical properties to critically examine the functional profile of the 149, as well as the 333 polymorphic variant of the human GLP-1R to further elucidate the potential contribution of these residues to receptor structure and function.

We have also shown in a previous study, that a SNP of the human GLP-1R that results in the conversion of Ser to Cys at amino acid residue 333, attenuated both the allosteric agonism as well as the modulatory properties of the Novo Nordisk small molecule, compound 2 (Koole et al., 2011). This effect was observed in the absence of any significant effects on peptide binding affinity or function as measured through cAMP accumulation, pERK1/2 and $_{i}Ca^{2+}$ mobilization outputs. In the current study, mutation of residue 333 to either Ala or Val did not significantly impact on peptide binding affinity or functional activity. Homology modeling of the GLP-1R using the glucagon receptor structure as a template places residue 333 in ICL3, close to the TM5/ICL3 boundary (Fig 1) (Siu et al., 2013). There is significant evidence to suggest that ICL3 is a major interaction interface for G proteins (Bavec et al., 2003), and this is illustrated in multiple mutagenesis studies (Heller et al., 1996; Mathi et al., 1997; Salapatek et al., 1999; Takhar et al., 1996), as well as in studies where G protein activation can by achieved with ICL3-corresponding peptides (Hallbrink et al., 2001). This work suggests an involvement of residues V327, I328 (Mathi et al., 1997), V331 (Mathi et al., 1997; Salapatek et al., 1999), I332, A333 (Salapatek et al., 1999), K334 (Mathi et al., 1997; Salapatek et al., 1999; Takhar et al., 1996) and R348 (Heller et al., 1996) in allowing effective G protein coupling to the GLP-1R. Notably, these mutagenesis data have been obtained using the rat GLP-1R. The human, mouse and rat GLP-1Rs share almost identical amino acid sequence in ICL3, suggesting that conservation of this region is important in the G protein coupling profile of the receptor across species. However, while S333 is conserved in both the human and mouse GLP-1R proteins, this residue is an Ala in the rat GLP-1R, consistent with our current data that indicate that this is not a critical residue in peptide-

mediated G protein coupling. These data may suggest that S333 lies outside the domain of critical importance in receptor-G protein interaction. In agreement, Mathi et al (1997) proposed that the N-terminal region of ICL3 is a helical projection of TM5 with S333 facing away from the G protein binding pocket, and this is supported by the glucagon receptor and CRF1 receptor crystal structures and in our homology model (Fig 1) (Hollenstein et al., 2013; Siu et al., 2013).

Despite little deviation in peptide function at the 333 residue in either the case of the polymorphic variant (C333; Koole et al., 2011) or the mutations introduced in this study (A333, V333), all these substitutions impact the cooperativity between compound 2 and oxyntomodulin, with no significant modulation of oxyntomodulin-mediated cAMP formation at C333 (Koole et al., 2011), and only weak positive modulation at the A333 and V333 mutants. However, unlike the C333 mutant that shows compromised compound 2-induced cAMP production in comparison to the wildtype GLP-1R (Koole et al, 2011), no significant change in cAMP production was observed at either the A333 or the V333 mutants. While the mechanistic basis of these observations is unclear, it suggests that there are potentially distinct receptor conformations stabilized by compound 2 that drive agonism versus cooperativity.

Recently, it has been revealed that compound 2 acts via covalent modification of the GLP-1R at C347 (located at the juxtaposition of ICL3/TM6; Fig 6), in a mechanism similar to that of the modulator, BETP (Nolte et al., 2014). Mutation of C347 to Ala resulted in loss of compound 2 agonism, in addition to loss of the PAM activity in the presence of GLP-1(9-36)NH_2, while peptide-mediated signaling was unaltered. It is possible that mutation of residue 333 impacts the neighboring interactions required for

compound 2 to covalently attach and/or induce conformations that favor sampling of receptor active states. Furthermore, the greater loss of compound 2 activity seen with the C333 mutant (Koole et al, 2011) may arise, at least in part, through alternate cross-linking through this site.

Unlike residue 333, most mutations of residue 149 resulted in significant attenuation of peptide binding and functional activity. The loss of binding affinity tracked through most functional assay systems assessed, manifesting as an attenuation of functional efficacy. Not surprisingly, mutation of 149 to Ser, that has similar chemical properties to that of the most frequently occurring residue, Thr, was relatively well tolerated and had the least influence on peptide binding and function in comparison to the other mutants. Nonetheless, significant reduction in affinity was observed with most peptides, with variable effects on signaling that suggest that even minor modification at the 149 position impact on receptor function.

Removal of the hydroxyl functional group from T149, with maintenance of chain length, by mutation to Val significantly reduced peptide binding affinity, and cAMP and $_{i}Ca^{2+}$ responses. Similarly, replacement of the hydroxyl group with a thiol group by means of mutation to Cys, significantly reduced peptide binding affinity that generally corresponded with decreases in cAMP and $_{i}Ca^{2+}$ outputs, albeit not always to significance. Interestingly, peptide-mediated pERK1/2 coupling efficacy (τ_c) at both V149 and C149 was not significantly altered. This is consistent with distinct mechanisms of conformational transition driving pERK1/2 versus cAMP/$_{i}Ca^{2+}$ signaling (Koole et al., 2012a; Koole et al., 2011; Wootten et al., 2013).

All other assessed mutations of residue 149 were poorly tolerated by the GLP-1R. Similar to that of the naturally occurring polymorphism M149, these residues are considerably more hydrophobic and bulky than the wildtype Thr, and consistent with M149, they have large detrimental effects. This is perhaps not surprising given the recent structural information that is emerging for the GLP-1R. Recently, Miller and colleagues have used photoaffinity labeling that detected close proximity between residues 16 and 12 of the GLP-1 peptide with residues 141 and 145 of the GLP-1R, respectively (Chen et al., 2010; Miller et al., 2011). Given that these residues precede, and would neighbor residue 149 in an alpha-helical tertiary structure, it indicates that 149 is near the endogenous peptide binding pocket. While not necessarily a ligand interaction point itself, as suggested by homology assessment with CRF1 and glucagon receptor TM structures (Fig 6) (Hollenstein et al., 2013; Siu et al., 2013), it may provide essential conformational restraints involving other TMs. This hypothesis is supported by recent alanine mutagenesis data of conserved polar residues in TM1 and TM2 where mutation of S155 (TM1) and S186 (TM2) differentially changed peptide-mediated signal bias at the receptor (Wootten et al., 2013). These residues are predicted to be involved in tight packing interactions with TM7 and TM3, respectively (Fig 6), and the data are consistent with a proximal role of residue 149 in the activation transition of the receptor following peptide binding.

Despite significant attenuation of peptide binding and function at almost all mutants of 149, the ability of compound 2 to signal via the receptor was, in most cases, minimally affected. Similar to our previous work showing that compound 2 was able to partially restore the binding affinity and cAMP response of M149, in this study we observed that

compound 2 was able to modulate almost all peptide responses when measured in cAMP. The most striking data observed here was the restoration of functional responses at the F149 and Y149 mutants that had severely abrogated cAMP signaling, demonstrating that compound 2 is able to lower the energy required for activation at receptor mutants that either have no detectable cAMP or very weakly stimulate a cAMP response. Given that antagonist binding was unaffected at any of these receptor mutants, this provides evidence for our previous hypothesis that residue 149 is involved in activation transition instead of direct disruption to peptide binding interactions (Koole et al., 2011).

Collectively, these results provide us with insight into domains that are essential for receptor function as well as the role that allosteric ligands play in receptor modulation.

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Authorship Contributions

Participated in research design: Koole, Wootten, Simms, Sexton

Conducted experiments: Koole

Contributed new reagents or analytic tools: N/A

Performed data analysis: Koole

Wrote or contributed to the writing of the manuscript: Koole, Wootten, Simms, Miller, Christopoulos, Sexton

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Footnotes

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Figure legends

Figure 1. Homology model of the GLP-1R based on the glucagon receptor structure. The positions of residues 149 and 333 are illustrated in red. The surface map of TM1 and TM2 helices is colored beige. TM5 and TM6 are depicted in green (ribbon structure). Inset: Magnification of ICL3 illustrating the position of residue 333 and C347 that is involved in covalent interaction with compound 2.

Figure 2. Characterization of the binding of GLP-1(1-36)NH_2 (A), GLP-1(7-36)NH_2 (B), Exendin-4 (C) and Oxyntomodulin (D) in competition with the radiolabeled antagonist, ^{125}I-exendin(9-39), in whole FlpInCHO cells stably expressing the wild type human GLP-1R or each of the human GLP-1R mutants at the 149 receptor residue. Data are normalized to maximum ^{125}I-exendin(9-39) binding, with nonspecific binding measured in the presence of 1 μM exendin(9-39), and analyzed with a three-parameter logistic equation as defined in Equation 1. X = 0 corresponds to conditions in which no ligand was added. All values are means ± S.E.M. of three independent experiments, conducted in duplicate.

Figure 3. Characterization of cAMP accumulation in the presence of GLP-1(1-36)NH_2 (A), GLP-1(7-36)NH_2 (B), Exendin-4 (C) and Oxyntomodulin (D) in FlpInCHO cells stably expressing the wild type human GLP-1R or each of the human GLP-1R mutants at the 149 receptor residue. Data are normalized to the response elicited by 100 μM forskolin and analyzed with an operational model of agonism as defined in Equation 2. X = 0 corresponds to conditions in which no ligand was added. All values are means ±

S.E.M. of four to five independent experiments, conducted in duplicate.

Figure 4. Characterization of $_{i}Ca^{2+}$ mobilization in the presence of GLP-1(7-36)NH_2 (A), Exendin-4 (B) and Oxyntomodulin (C) in FlpInCHO cells stably expressing the wild type human GLP-1R or each of the human GLP-1R mutants at the 149 receptor residue. Data are normalized to the response elicited by 100 μM ATP and analyzed with an operational model of agonism as defined in Equation 2 (A and B). X = 0 corresponds to conditions in which no ligand was added. Data presented in (C) are levels of $_{i}Ca^{2+}$ mobilization in the presence of 1 μM oxyntomodulin and are normalized to the maximal response elicited by 100 μM ATP. Statistical significance of changes in response in comparison with wildtype human GLP-1R were determined by one-way analysis of variance and Dunnett's post-test and are indicated with an asterisk (*, $p < 0.05$). All values are mean ± S.E.M. of five to seven independent experiments, conducted in duplicate.

Figure 5. Characterization of pERK1/2 in the presence of GLP-1(1-36)NH_2 (A), GLP-1(7-36)NH_2 (B), Exendin-4 (C) and Oxyntomodulin (D) in FlpInCHO cells stably expressing the wild type human GLP-1R or each of the human GLP-1R 149 mutants. Data are normalized to the maximal response elicited by 10% FBS and analyzed with an operational model of agonism as defined in Equation 2. X = 0 corresponds to conditions in which no ligand was added. All values are mean ± S.E.M. of four to six independent experiments, conducted in duplicate.

Figure 6. Homology model of the GLP-1R illustrating the location of T149 (red, cpg representation) in relation to L141 and Y145 that are reported to be in close proximity to GLP-1 peptide residues 16 and 12, respectively (Chen et al., 2010; Miller et al., 2011; blue, ball and stick representation), and S155 (TM1) and S186 (TM2) that are involved in peptide-mediated signal bias (Wootten et al., 2013).

Table 1. Effects of human GLP-1R 149 or 333 mutation on peptide ligand binding and cell surface expression.

Binding data were analyzed using a three-parameter logistic equation as defined in Equation 1 to obtain pIC_{50} values. pIC_{50} values were then corrected for radioligand occupancy using the radioligand dissociation constant for each mutant, allowing determination of ligand affinity (K_i). Data were normalized to maximum ^{125}I-exendin(9-39) binding in the absence of ligand, with nonspecific binding measured in the presence of 1 μM exendin(9-39). For specific ^{125}I-exendin(9-39) binding, data are expressed as a maximum of specific ^{125}I-exendin(9-39) binding at the wildtype human GLP-1R. Cell surface expression was determined through antibody detection of the N-terminal c-myc epitope label, with data expressed as a maximum of wildtype human GLP-1R expression. All values are expressed as mean ± S.E.M. of three to four independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	Binding (pK_i)				**Cell surface expression (% wildtype)**	**Specific ^{125}I-exendin(9-39) binding (% wildtype)**
	GLP-1(7-36)NH_2	Exendin-4	Oxyntomodulin	Exendin(9-39)□		
Wildtype (T^{149}, S^{333})	9.1 ± 0.1	9.7 ± 0.1	8.1 ± 0.1	8.0 ± 0.0	100 ± 6	100 ± 14
M^{149}§	**6.5 ± 0.2***	**7.9 ± 0.2***	**5.5 ± 0.2***	**N.A.**	**47 ± 9***	**N.A.**
A^{149}	**6.8 ± 0.1***	**7.8 ± 0.1***	**6.2 ± 0.1***	8.0 ± 0.0	110 ± 7	113 ± 30
C^{149}	**7.6 ± 0.1***	**8.4 ± 0.1***	**6.9 ± 0.1***	8.0 ± 0.0	**156 ± 6***	218 ± 29#
F^{149}	**6.4 ± 0.1***	**7.5 ± 0.1***	**5.6 ± 0.1***	8.0 ± 0.0	94 ± 6	90 ± 23
I^{149}	**6.8 ± 0.1***	**7.8 ± 0.1***	**6.0 ± 0.2***	8.0 ± 0.0	99 ± 5	154 ± 20
S^{149}	**8.3 ± 0.1***	9.4 ± 0.0	**7.6 ± 0.1***	8.0 ± 0.0	88 ± 7	125 ± 24
V^{149}	**6.7 ± 0.1***	**7.8 ± 0.1***	**6.2 ± 0.1***	8.0 ± 0.0	**72 ± 4***	139 ± 26
Y^{149}	**6.8 ± 0.1***	**7.5 ± 0.1***	**6.0 ± 0.1***	8.0 ± 0.0	**53 ± 5***	87 ± 15
C^{333}§	9.0 ± 0.1	9.6 ± 0.1	8.0 ± 0.1	**N.A.**	**51 ± 6***	**N.A.**
A^{333}	9.1 ± 0.1	9.6 ± 0.1	8.0 ± 0.1	8.0 ± 0.0	122 ± 9	124 ± 15
V^{333}	9.2 ± 0.1	9.8 ± 0.1	8.4 ± 0.1	8.0 ± 0.0	86 ± 5	101 ± 26

* statistically significant at $p < 0.05$, one-way analysis of variance and Dunnett's post test in comparison to wildtype control

□ equivalent at one significant figure

$p = 0.11$

§ data obtained from Koole et al., 2011. Reported values for binding are pIC_{50}

N.A. data not experimentally determined in Koole et al., 2011

Table 2. Effects of human GLP-1R 149 or 333 mutation on agonist signaling via cAMP.

Data were analyzed using a three-parameter logistic equation as defined in Equation 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that elicited by 100 μM forskolin. All mutants were analyzed with an operational model of agonism (Equation 2) to determine logτ values. All logτ values were then corrected to specific ^{125}I-exendin(9-39) binding ($log\tau_c$). Values are expressed as mean ± S.E.M. of four to five independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	cAMP accumulation														
	GLP-1(1-36)NH_2			GLP-1(7-36)NH_2			Exendin-4			Oxyntomodulin			Compound 2		
	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)
Wildtype	7.4 ± 0.1	149.7 ± 7.8	0.53 ± 0.16 (3.36)	9.4 ± 0.2	175.7 ± 10.1	0.94 ± 0.22 (8.63)	10.5 ± 0.1	162.5 ± 5.4	0.70 ± 0.19 (5.05)	8.5 ± 0.1	178.3 ± 8.8	1.03 ± 0.24 (10.74)	6.0 ± 0.2	115.4 ± 9.2	0.18 ± 0.17 (1.51)
M^{149}§	**N.D.**	**N.D.**	**N.A.**	**8.0 ± 0.3***	**N.D.**	**N.A.**	**8.4 ± 0.1***	223 ± 13	**N.A.**	**N.D.**	**N.D.**	**N.A.**	5.5 ± 0.1	217 ± 24	**N.A.**
A^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**8.9 ± 0.2***	139.1 ± 11.0	0.34 ± 0.33 (2.20)	**N.D.**	**N.D.**	**N.D.**	5.7 ± 0.4	**70.2 ± 10.4***	-0.27 ± 0.32 (0.53)
C^{149}	6.8 ± 0.2	44.7 ± 4.9	-0.86 ± 0.32 (0.14)	8.4 ± 0.2	123.5 ± 10.2	-0.10 ± 0.31 (0.79)	9.8 ± 0.2	133.3 ± 10.0	0.00 ± 0.30 (1.00)	**7.1 ± 0.1***	**120.6 ± 7.0***	-0.13 ± 0.31 (0.74)	5.7 ± 0.3	81.7 ± 9.9	-0.46 ± 0.30 (0.35)
F^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**8.0 ± 0.1***	150.6 ± 9.5	0.57 ± 0.33 (3.75)	**N.D.**	**N.D.**	**N.D.**	5.9 ± 0.3	82.9 ± 9.6	-0.06 ± 0.25 (0.87)
I^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**8.2 ± 0.1***	**86.6 ± 6.6***	-0.28 ± 0.26 (0.52)	**N.D.**	**N.D.**	**N.D.**	6.1 ± 0.3	83.7 ± 7.8	-0.29 ± 0.22 (0.51)
S^{149}	6.8 ± 0.1	170.1 ± 9.5	0.74 ± 0.34 (5.52)	9.1 ± 0.2	129.5 ± 9.4	0.20 ± 0.25 (1.57)	10.3 ± 0.2	186.0 ± 12.4	1.23 ± 0.50 (17.02)	7.9 ± 0.2	141.7 ± 9.1	0.33 ± 0.26 (2.13)	5.9 ± 0.2	80.8 ± 9.9	-0.23 ± 0.25 (0.59)
V^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**8.9 ± 0.3***	122.1 ± 13.0	0.08 ± 0.29 (1.21)	**N.D.**	**N.D.**	**N.D.**	6.0 ± 0.4	105.9 ± 12.3	-0.05 ± 0.27 (0.90)
Y^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**8.1 ± 0.2***	**77.5 ± 10.5***	-0.13 ± 0.22 (0.74)	**N.D.**	**N.D.**	**N.D.**	5.8 ± 0.3	**41.6 ± 4.9***	-0.46 ± 0.19 (0.34)
C^{333}§	6.9 ± 0.2	259 ± 24	**N.A.**	10.2 ± 0.3	331 ± 26	**N.A.**	10.2 ± 0.2	257 ± 12	**N.A.**	8.2 ± 0.2	288 ± 16	**N.A.**	**N.D.**	**46 ± 8***§§	**N.A.**
A^{333}	7.4 ± 0.1	142.9 ± 9.7	0.35 ± 0.17 (2.25)	9.5 ± 0.1	166.7 ± 8.6	0.68 ± 0.19 (4.82)	10.5 ± 0.2	168.2 ± 11.7	0.71 ± 0.21 (5.15)	8.4 ± 0.2	162.7 ± 9.4	0.61 ± 0.19 (4.09)	5.8 ± 0.2	142.7 ± 13.0	0.36 ± 0.19 (2.29)
V^{333}	7.2 ± 0.1	112.1 ± 8.7	0.13 ± 0.27 (1.36)	9.5 ± 0.2	120.8 ± 8.2	0.21 ± 0.27 (1.63)	10.5 ± 0.2	132.4 ± 7.1	0.33 ± 0.27 (2.13)	8.6 ± 0.2	**97.5 ± 7.2***	0.00 ± 0.27 (1.00)	5.9 ± 0.2	100.9 ± 9.6	0.05 ± 0.28 (1.12)

N.D., data unable to be experimentally defined or with incomplete curves

* statistically significant at $p < 0.05$, one-way analysis of variance and Dunnett's post test in comparison to wildtype control

§ data obtained from Koole et al., 2011. Reported E_{max} values are normalized to 100 nM forskolin

§§ compound 2 response at 10^{-5}M

N.A. data not experimentally determined in Koole et al., 2011

Table 3. Effects of human GLP-1R 149 or 333 mutation on agonist signaling via $_{i}Ca^{2+}$ mobilization.

Data were analyzed using a three-parameter logistic equation as defined in Equation 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that elicited by 100 μM ATP. All mutants were analyzed with an operational model of agonism (Equation 2) to determine logτ values. All logτ values were then corrected to specific ^{125}I-exendin(9-39) binding ($log\tau_c$). Values are expressed as mean ± S.E.M. of five to seven independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	$_{i}Ca^{2+}$ mobilization						
	GLP-1(7-36)NH_2			Exendin-4			Oxyntomodulin
	pEC_{50}	E_{max}	Logτ_c (τ_c)	pEC_{50}	E_{max}	Logτ_c (τ_c)	E_{max}□
Wildtype	7.6 ± 0.2	14.3 ± 1.3	0.08 ± 0.16 (1.20)	7.3 ± 0.2	17.3 ± 1.5	0.29 ± 0.16 (1.95)	10.8 ± 1.8
M^{149}§	**N.D.**	**N.D.**	**N.A.**	**N.D.**	**N.D.**	**N.A**	**2.6 ± 0.4***
A^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
C^{149}	7.2 ± 0.1	11.9 ± 0.8	-0.42 ± 0.3 (0.38)	7.5 ± 0.3	**6.1 ± 0.8***	**-0.86 ± 0.31 (0.14)***	**3.1 ± 2.6***
F^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
I^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
S^{149}	7.1 ± 0.2	17.9 ± 2.2	0.23 ± 0.26 (1.70)	7.1 ± 0.2	15.5 ± 1.7	0.05 ± 0.26 (1.12)	5.2 ± 2.4
V^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
Y^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**
C^{333}§	7.8 ± 0.3	23.6 ± 2.4	**N.A.**	7.8 ± 0.2	**14.6 ± 1.1***	**N.A.**	15.0 ± 2.4
A^{333}	7.8 ± 0.2	**19.1 ± 1.4***	0.35 ± 0.17 (2.24)	7.4 ± 0.2	19.3 ± 1.7	0.36 ± 0.18 (2.29)	13.3 ± 2.3
V^{333}	7.8 ± 0.2	17.4 ± 1.6	0.30 ± 0.27 (2.00)	7.6 ± 0.2	18.7 ± 1.7	0.40 ± 0.27 (2.51)	15.9 ± 3.2

N.D., data unable to be experimentally defined or with incomplete curves

* statistically significant at $p < 0.05$, one-way analysis of variance and Dunnett's post test in comparison to wildtype control

□ response at 1 μM oxyntomodulin

§ data obtained from Koole et al., 2011. Reported E_{max} values are normalized to 100 μM forskolin

N.A. data not experimentally determined in Koole et al., 2011

Table 4. Effects of human GLP-1R 149 or 333 mutation on agonist signaling via pERK1/2.

Data were analyzed using a three-parameter logistic equation as defined in Equation 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that elicited by 10% FBS. All mutants were analyzed with an operational model of agonism (Equation 2) to determine logτ values. All logτ values were then corrected to specific ^{125}I-exendin(9-39) binding (logτ_c). Values are expressed as mean ± S.E.M. of four to six independent experiments, conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	pERK1/2														
	GLP-1(1-36)NH_2			GLP-1(7-36)NH_2			Exendin-4			Oxyntomodulin			Compound 2		
	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)	pEC_{50}	E_{max}	$Log\tau_c$ (τ_c)
Wildtype	7.3 ± 0.2	1.3 ± 0.1	-0.72 ± 0.16 (0.19)	8.0 ± 0.2	3.6 ± 0.2	-0.10 ± 0.15 (0.79)	8.1 ± 0.2	4.2 ± 0.3	0.02 ± 0.16 (1.05)	7.6 ± 0.1	5.0 ± 0.2	0.21 ± 0.16 (1.62)	5.9 ± 0.2	0.45 ± 0.1	-1.21 ± 0.19 (0.06)
M^{149}§	**N.D.**	0.6 ± 0.1	**N.A.**	7.8 ± 0.3	4.1 ± 0.5	**N.A.**	8.0 ± 0.2	**3.4 ± 0.3***	**N.A.**	7.1 ± 0.2	**3.7 ± 0.3***	**N.A.**	5.9 ± 0.3	2.0 ± 0.4	**N.A.**
A^{149}	**N.D.**	**N.D.**	**N.D.**	6.9 ± 0.4	**1.7 ± 0.4***	-0.58 ± 0.41 (0.26)	7.6 ± 0.4	3.0 ± 0.5	-0.30 ± 0.32 (0.50)	7.0 ± 0.2	**2.6 ± 0.2***	-0.38 ± 0.32 (0.42)	4.7 ± 0.7	0.99 ± 0.7	-0.95 ± 0.38 (0.11)
C^{149}	6.4 ± 0.5	1.6 ± 0.6	-0.90 ± 0.43 (0.13)	7.3 ± 0.4	**2.0 ± 0.4***	-0.82 ± 0.31 (0.15)	7.7 ± 0.3	4.0 ± 0.4	-0.36 ± 0.31 (0.44)	6.9 ± 0.2	**3.3 ± 0.4***	-0.48 ± 0.31 (0.33)	5.6 ± 0.4	0.33 ± 0.1	-1.67 ± 0.38 (0.02)
F^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	7.7 ± 0.2	**1.3 ± 0.1***	-0.71 ± 0.32 (0.19)	7.1 ± 0.2	**1.3 ± 0.1***	-0.64 ± 0.28 (0.23)	4.8 ± 0.4	0.87 ± 0.4	-0.78 ± 0.45 (0.17)
I^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	7.6 ± 0.3	**1.1 ± 0.2***	-1.02 ± 0.39 (0.10)	7.3 ± 0.2	**1.3 ± 0.1***	-0.91 ± 0.26 (0.12)	5.2 ± 0.4	0.71 ± 0.2	-1.21 ± 0.26 (0.06)
S^{149}	6.8 ± 0.4	1.4 ± 0.3	-0.82 ± 0.26 (0.15)	7.5 ± 0.2	3.3 ± 0.4	-0.27 ± 0.25 (0.54)	7.9 ± 0.2	3.7 ± 0.3	-0.19 ± 0.26 (0.65)	7.3 ± 0.2	4.9 ± 0.5	0.09 ± 0.25 (1.23)	5.2 ± 0.4	0.79 ± 0.2	-1.04 ± 0.29 (0.09)
V^{149}	**N.D.**	**N.D.**	**N.D.**	7.5 ± 0.6	**1.1 ± 0.3***	-0.97 ± 0.34 (0.11)	7.2 ± 0.8	**0.8 ± 0.4***	-1.12 ± 0.95 (0.08)	7.1 ± 0.3	**1.5 ± 0.3***	-0.77 ± 0.31 (0.17)	5.5 ± 0.6	0.29 ± 0.1	-1.50 ± 0.42 (0.03)
Y^{149}	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	**N.D.**	6.7 ± 0.6	**1.0 ± 0.3***	-0.85 ± 0.67 (0.14)	7.0 ± 0.5	**0.6 ± 0.2***	**-1.02 ± 0.36 (0.10)***	4.8 ± 0.8	0.49 ± 0.4	-1.15 ± 0.41 (0.07)
C^{333}§	7.7 ± 0.2	1.7 ± 0.2	**N.A.**	8.7 ± 0.2	5.9 ± 0.4	**N.A.**	9.1 ± 0.3	6.1 ± 0.5	**N.A.**	7.7 ± 0.2	7.0 ± 0.5	**N.A.**	6.0 ± 0.2	1.4 ± 0.2	**N.A.**
A^{333}	7.0 ± 0.2	1.7 ± 0.2	-0.66 ± 0.17 (0.22)	7.8 ± 0.2	**6.1 ± 0.6***	0.40 ± 0.18 (2.51)	8.0 ± 0.3	**6.7 ± 0.8***	0.60 ± 0.21 (3.98)	7.5 ± 0.2	**7.5 ± 0.7***	1.07 ± 0.36 (11.75)	6.2 ± 0.3	0.70 ± 0.1	-1.10 ± 0.17 (0.08)
V^{333}	7.0 ± 0.2	1.4 ± 0.2	-0.70 ± 0.28 (0.20)	7.8 ± 0.1	4.1 ± 0.2	0.02 ± 0.27 (1.05)	8.0 ± 0.1	4.7 ± 0.3	0.13 ± 0.27 (1.35)	7.6 ± 0.2	6.3 ± 0.5	0.57 ± 0.28 (3.72)	5.9 ± 0.4	1.11 ± 0.2	-0.79 ± 0.27 (0.16)

N.D., data unable to be experimentally defined or with incomplete curves

* statistically significant at $p < 0.05$, one-way analysis of variance and Dunnett's post test in comparison to wildtype control

§ data obtained from Koole et al., 2011. Reported E_{max} values are normalized to 10% FBS

N.A. data not experimentally determined in Koole et al., 2011

Table 5. Differential modulation of agonist peptides at human GLP-1R 149 or 333 mutants by compound 2 in cAMP accumulation.

Data were analyzed using a three-parameter logistic equation as defined in Equation 1. pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. All data are normalized to the response elicited by 100 μM forskolin and then normalized to the response elicited by 1 μM peptide (GLP-1(1-36)NH_2, oxyntomodulin) or 100 nM peptide (GLP-1(7-36)NH_2, exendin-4) at each mutant. Values are expressed as mean ± S.E.M. of four to nine independent experiments, conducted in duplicate.

	cAMP accumulation (pEC_{50})							
	GLP-1(1-36)NH_2	+ 3 µM compound 2	GLP-1(7-36)NH_2	+ 3 µM compound 2	Exendin-4	+ 3 µM compound 2	Oxyntomodulin	+ 3 µM compound 2
Wildtype	7.8 ± 0.1	7.5 ± 0.4	10.6 ± 0.1	10.9 ± 0.5	11.1 ± 0.1	10.8 ± 0.8	9.5 ± 0.1	**10.4 ± 0.8***
M^{149}§	**N.D.**	**N.D.**	7.0 ± 0.2	**9.7 ± 0.8***	8.5 ± 0.2	**9.6 ± 0.7***	**N.D.**	**7.7 ± 1.5***
A^{149}	7.0 ± 0.1	**7.8 ± 0.8***	8.7 ± 0.1	**10.5 ± 0.2***	9.9 ± 0.1	**10.7 ± 0.4***	7.6 ± 0.1	**9.6 ± 0.2***
C^{149}	7.2 ± 0.1	7.1 ± 0.7	9.3 ± 0.1	10.2 ± 0.5	10.6 ± 0.2	11.0 ± 0.9	8.0 ± 0.1	**9.5 ± 0.8***
F^{149}	**N.D.**	7.2 ± 1.4	**N.D.**	10.0 ± 0.2	8.8 ± 0.1	**11.1 ± 0.5***	7.3 ± 0.2	**8.5 ± 0.7***
I^{149}	**N.D.**	**N.D.**	**N.D.**	8.9 ± 0.5	8.8 ± 0.0	**9.9 ± 0.6***	**N.D.**	7.7 ± 3.1
S^{149}	7.4 ± 0.1	7.4 ± 0.5	10.4 ± 0.2	10.9 ± 0.8	11.0 ± 0.1	11.3 ± 0.5	9.0 ± 0.1	**10.0 ± 0.4***
V^{149}	**N.D.**	7.3 ± 1.6	8.5 ± 0.0	**N.D.**	9.8 ± 0.1	9.8 ± 0.4	7.3 ± 0.1	**8.7 ± 0.5***
Y^{149}	**N.D.**	7.4 ± 0.7	**N.D.**	10.0 ± 0.3	8.7 ± 0.1	**10.8 ± 0.2***	**N.D.**	8.3 ± 0.5
C^{333}§	**N.A.**	**N.A.**	**N.A.**	**N.A.**	**N.A.**	**N.A.**	8.2 ± 0.1	8.3 ± 0.2
A^{333}	7.6 ± 0.1	7.5 ± 0.8	10.5 ± 0.2	10.3 ± 0.5	11.2 ± 0.1	11.2 ± 0.5	9.4 ± 0.1	9.6 ± 0.4
V^{333}	8.0 ± 0.1	7.8 ± 0.8	10.6 ± 0.1	10.7 ± 0.5	11.1 ± 0.1	11.6 ± 0.4	9.5 ± 0.1	10.0 ± 0.5

N.D., data unable to be experimentally defined or with incomplete curves

* statistically significant at $p \leq 0.05$, compared with respective peptide control, paired *t* test

§ data obtained from Koole et al., 2011. Reported values are normalized to 100 nM forskolin

N.A. data not experimentally determined in Koole et al., 2011

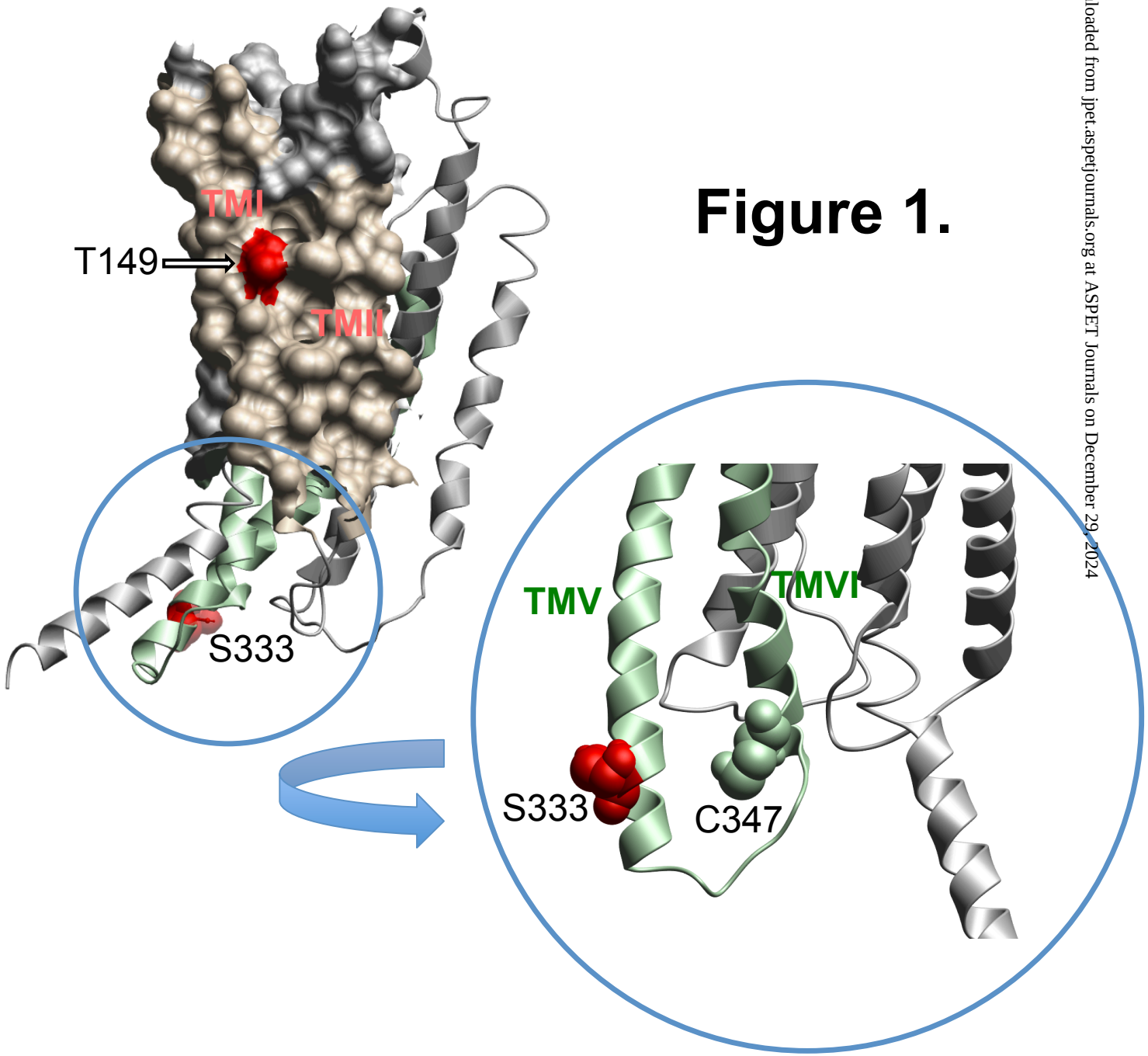

Figure 1.

JPET Fast Forward. Published on January 28, 2015 as DOI: 10.1124/jpet.114.220913
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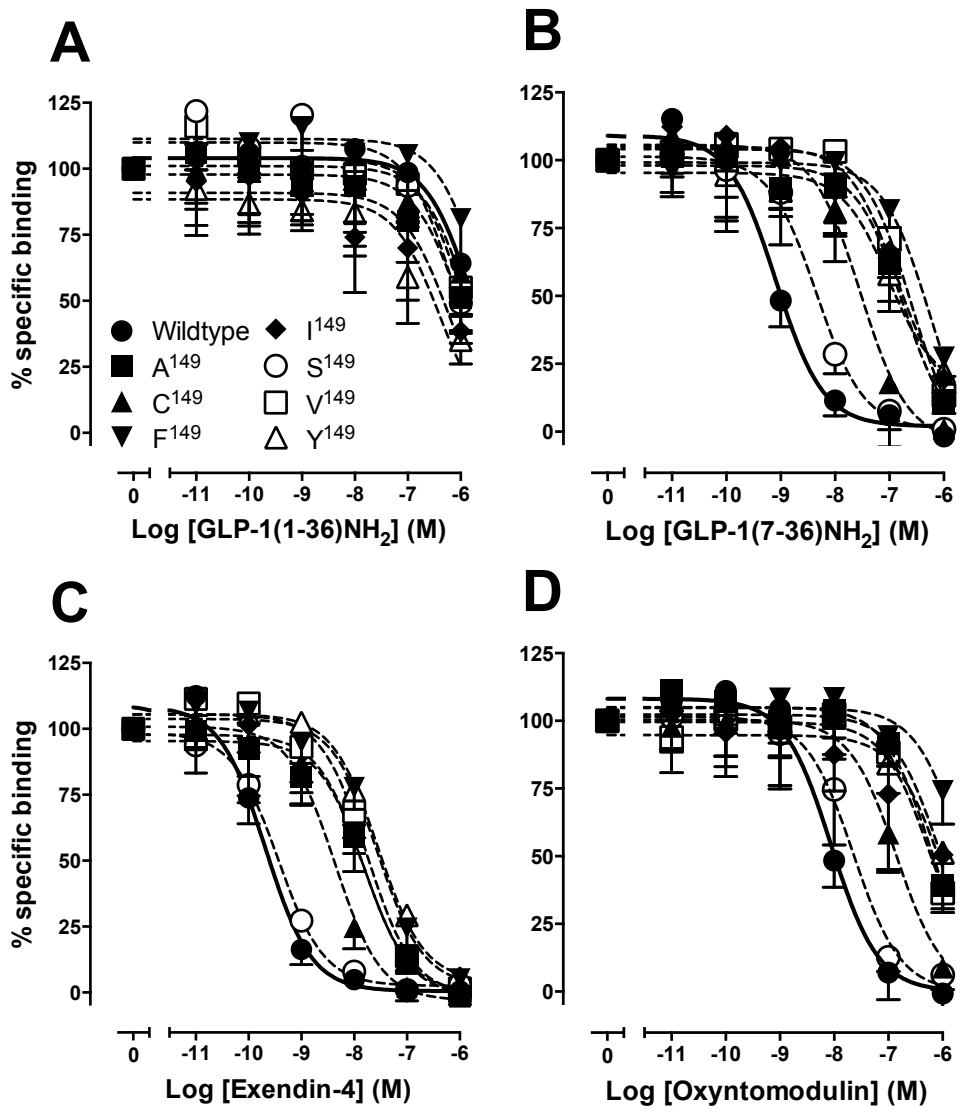

Figure 2.

JPET Fast Forward. Published on January 28, 2015 as DOI: 10.1124/jpet.114.220913

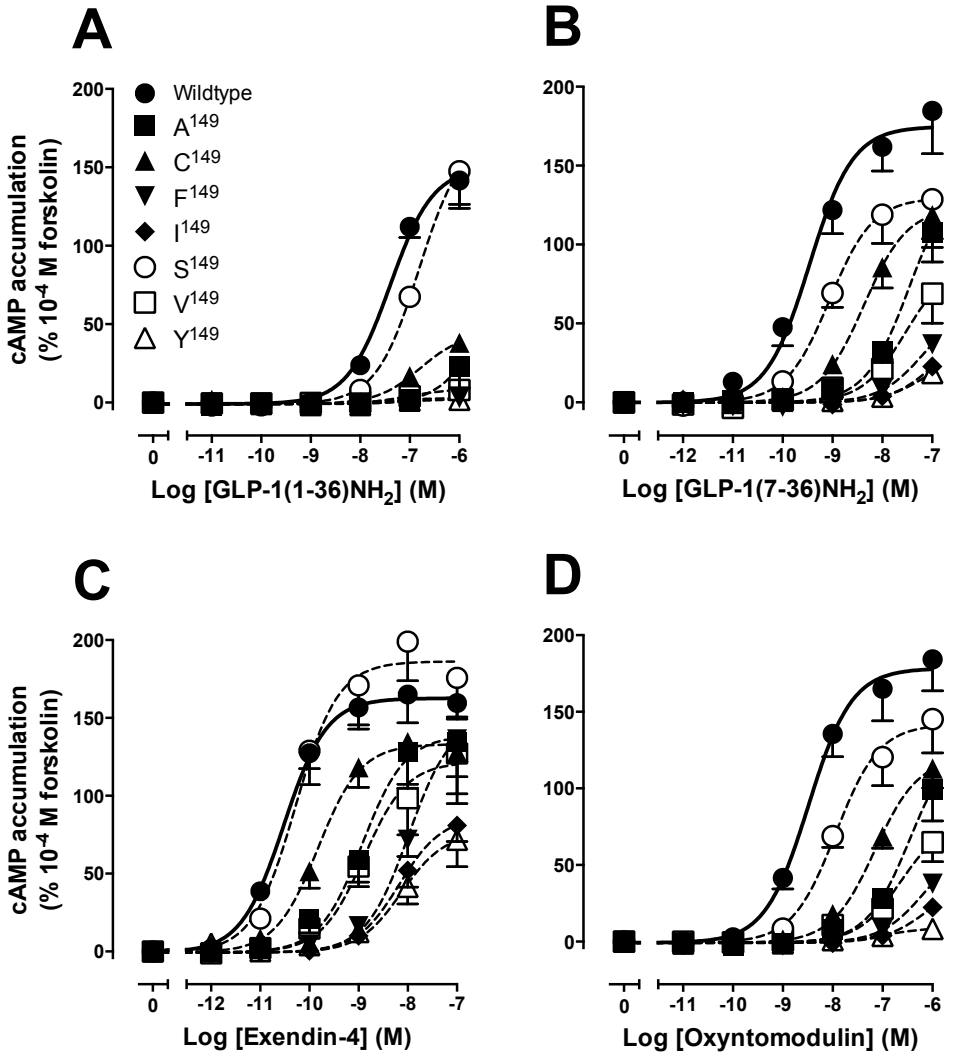

Figure 3.

JPET Fast Forward. Published on January 28, 2015 as DOI: 10.1124/jpet.114.220913

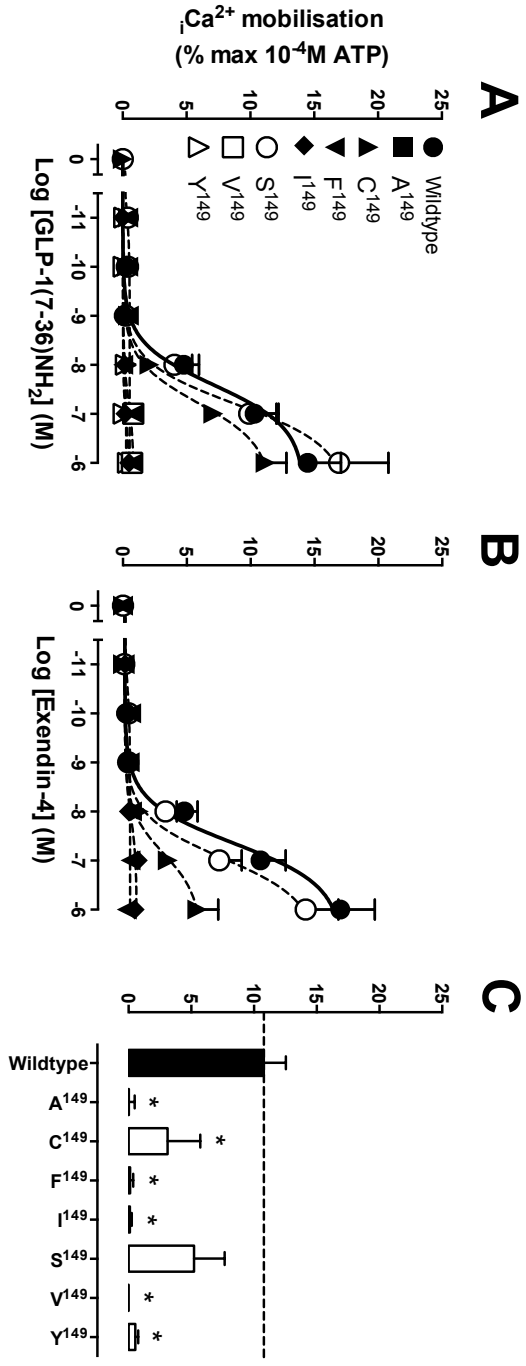

Figure 4.

JPET Fast Forward. Published on January 28, 2015 as DOI: 10.1124/jpet.114.220913

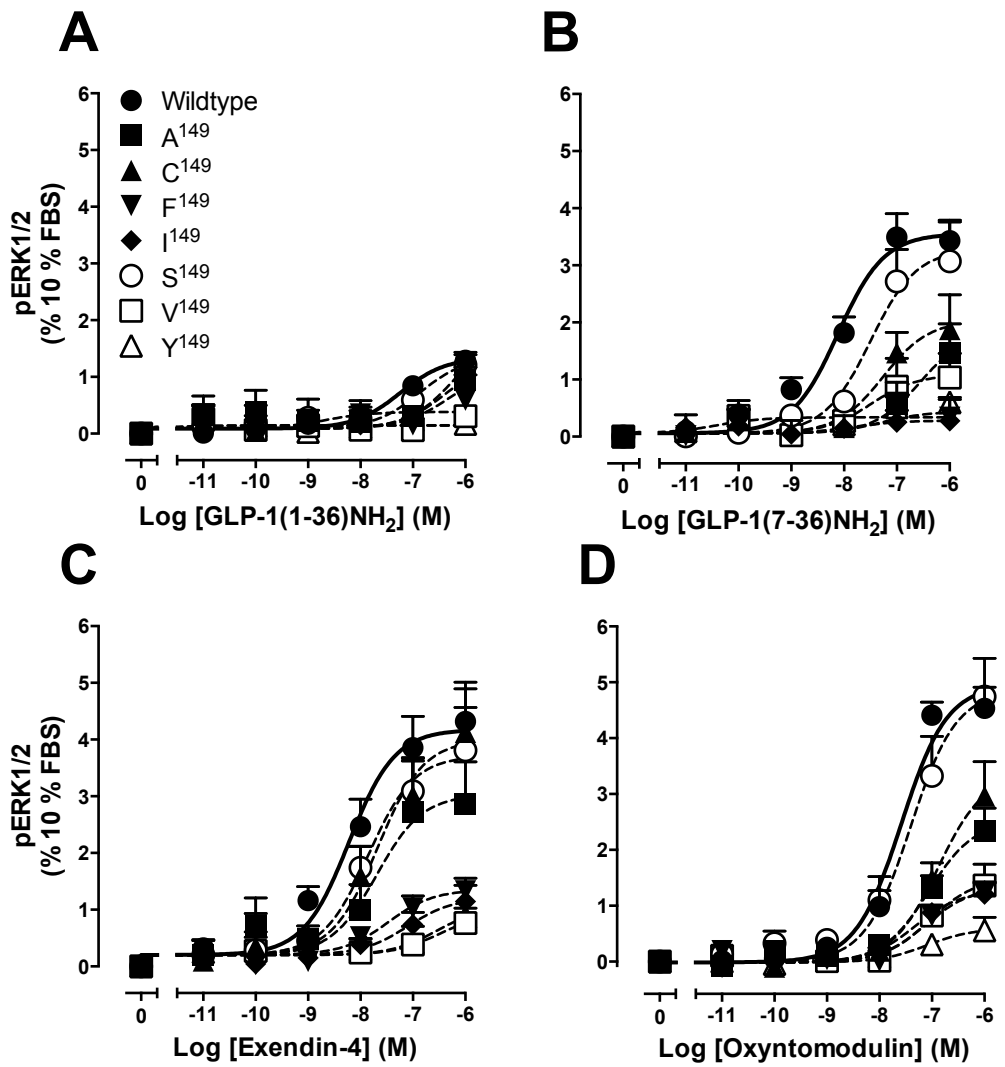

Figure 5.

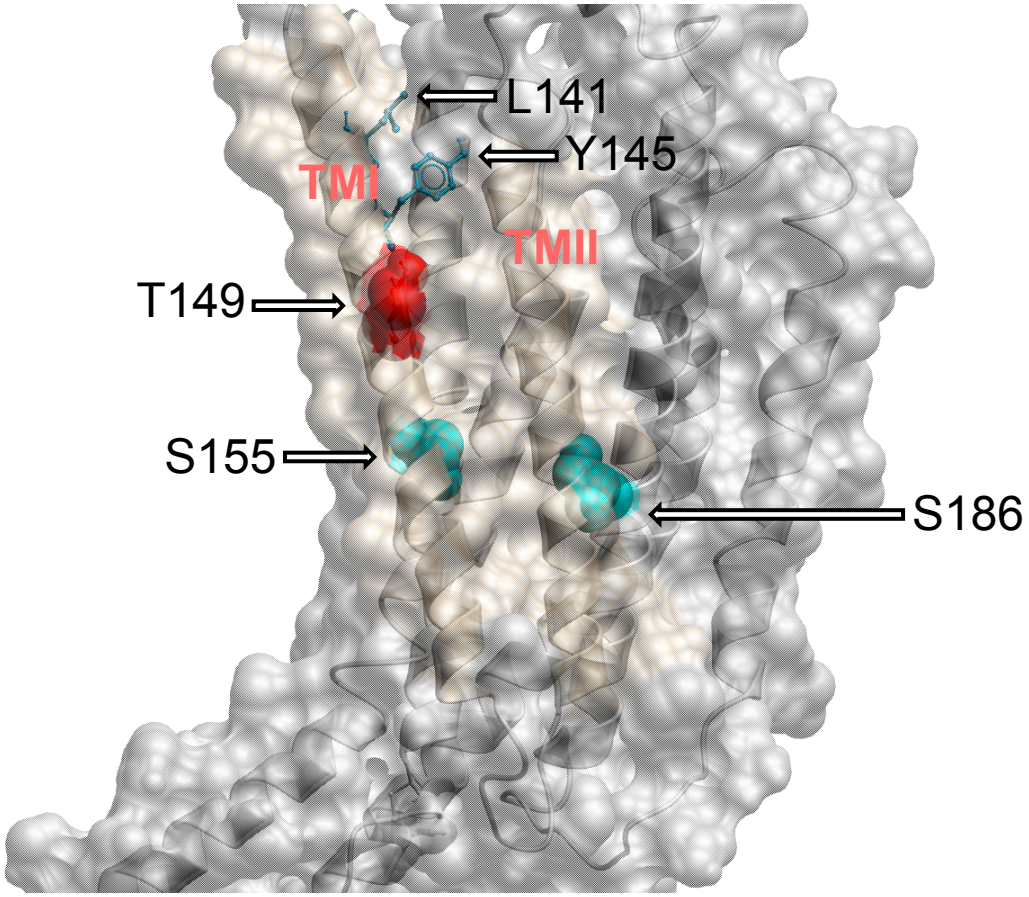

Figure 6.