Molecular Pharmacology Fast Forward. Published on June 14, 2010 as doi:10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner; implications for drug screening

Cassandra Koole[1], Denise Wootten[1], John Simms[1], Celine Valant, Rohan Sridhar, Owen L Woodman, Laurence J Miller, Roger J Summers, Arthur Christopoulos and Patrick M. Sexton

Drug Discovery Biology Laboratory, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria 3052, Australia (CK, DW, CV, AC, RJS, RS, JS, PMS), Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ 85259, USA (LJM) and School of Medical Sciences, RMIT University, Bundoora, Victoria 3083, Australia (OLW).

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Differential allosteric modulation of the GLP-1R

Text pages: 37

Number of figures: 5

Number of tables: 3

Number of references: 42

Number of words in Abstract: 249

Number of words in Introduction: 789

Number of words in Discussion: 1584

Abbreviations

ACh - acetylcholine, ACTH - adrenocorticotropic hormone, AgRP - agouti-related peptide, α-MSH - α-melanocyte stimulating hormone, BCA - bicinchoninic acid, BSA - bovine serum albumin, CHO - Chinese hamster ovary, DM - diabetes mellitus, DMEM - Dulbeccos modified eagles medium, DPPIV - dipepidyl peptidase IV, EPAC - exchange protein directly activated by cAMP, ERK1/2 - extracellular signal-regulated kinases 1 and 2, FBS - fetal bovine serum, Gα - α subunit of G protein, GLP-1R - glucagon-like peptide 1 receptor, GPCR - G protein-coupled receptor, IBMX - 3-Isobutyl-1-methylxanthine, MAPK - mitogen activated protein kinases, PEI - polyethylenimine, PI3 - phosphoinositide 3, PKA - protein kinase A, PTH - parathyroid hormone, PTHrP - parathyroid hormone-related protein

Address correspondence to:

Prof. Patrick M. Sexton, Drug Discovery Biology Laboratory, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. Email: Patrick.sexton@med.monash.edu.au

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The glucagon-like peptide-1 (GLP-1) receptor is a key regulator of insulin secretion and a major therapeutic target for treatment of diabetes. However, GLP-1 receptor function is complex with multiple endogenous peptides that can interact with the receptor, including full length (1-37) and truncated (7-37) forms of GLP-1 that can each exist in an amidated form, and the related peptide oxyntomodulin. We have investigated two GLP-1 receptor allosteric modulators, Novo Nordisk compound 2 and quercetin, and their ability to modify binding and signalling (cAMP formation, intracellular Ca^{2+} mobilisation and ERK1/2 phosphorylation) of each of the naturally occurring endogenous peptide agonists, as well as the clinically used peptide mimetic, exendin-4. We identified and quantified stimulus bias across multiple endogenous peptides, with response profiles for truncated GLP-1 peptides distinct from those of either the full-length GLP-1 peptides or oxyntomodulin; the first demonstration of such behaviour at the GLP-1 receptor. Compound 2 selectively augmented cAMP signalling but did so in a peptide-agonist dependent manner having greatest effect on oxyntomodulin, weaker effect on truncated GLP-1 peptides and negligible effect on other peptide responses; these effects were principally driven by parallel changes in peptide agonist affinity. In contrast, quercetin selectively modulated calcium signalling but with effects only on truncated GLP-1 peptides or exendin and not oxyntomodulin or full-length peptides. These data have significant implications for how GLP-1 receptor targeted drugs are screened and developed, while the allosterically driven, agonist-selective, stimulus bias highlights the potential for distinct clinical efficacy depending on the properties of individual drugs.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Type II (non-insulin-dependent) diabetes mellitus (DM) is a major disease of the western world with many complications including renal failure and vascular conditions leading to heart attack and stroke (Wild et al., 2004). Type II DM is characterised by a decrease in peripheral tissue response to insulin in association with impaired β cell function, which results in an increase in fasting glycemia (DeFronzo, 1992). Whilst anti-hyperglycaemic drugs such as metformins, sulfonylureas or thiazolidinediones may be prescribed to promote insulin secretion or enhance insulin sensitivity (Mitri and Hamdy, 2009), these drugs do not target all of the symptoms of type II DM. In recent years, drugs that enhance the activity of the glucagon-like peptide 1 receptor (GLP-1R) have been of particular interest to the pharmaceutical industry, as activation of this receptor addresses most of the manifestations of the condition.

The GLP-1R is a Family B peptide hormone G protein-coupled receptor (GPCR) primarily expressed in pancreatic β cells, and responds to at least four distinct endogenous GLP-1 variants, as well as to the related peptide oxyntomodulin and exogenous mimetic peptides such as exendin-4. The four secreted forms of GLP-1 include a full length peptide GLP-1(1-37) and a truncated form GLP-1(7-37), each of which also has an amidated counterpart, GLP-1(1-36)NH_2 and GLP-1(7-36)NH_2, respectively (Baggio & Drucker, 2007; Estall & Drucker, 2006). Although levels of GLP-1 are reduced in type II DM patients, the receptor retains insulinotropic properties (Toft-Nielsen et al., 2001). However, the promise of this receptor as a target in the development of type II DM is hindered by the rapid degradation of endogenous peptides by dipeptidyl peptidase IV (DPPIV) *in vivo* (Deacon et al., 1995a; Kieffer et al., 1995). This has in part been overcome by the development of DPPIV resistant GLP-1 mimetics such as exendin-4 (Edwards et al., 2001; Goke et al., 1993) and liraglutide (Elbrond et al., 2002; Knudsen et al., 2000), as well as DPPIV inhibitors that prolong the plasma half-

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

life of endogenous GLP-1R peptides (Deacon et al., 1995b). Although these have therapeutic potential and indeed exendin-4 is currently used clinically, they require frequent intravenous or subcutaneous administration, reducing compliance. In addition, exendin-4 has also been associated with significant adverse side effects in some patients, including pancreatitis (Olansky, 2010), of which the mechanistic basis is unknown. These difficulties have therefore driven the search for the development of small molecule orally active drugs that augment GLP-1R signalling.

Allosteric ligands bind to GPCRs at sites distinct from the orthosteric (endogenous agonist) binding site, and can modulate binding and/or signalling pathways of the receptor, as well as potentially acting as agonists themselves. Allosteric modulation has recently gained much traction as a means to overcome the limitations of many orthosterically targeted ligands, as they have the ability to provide novel receptor specificity and selectively control receptor function (Christopoulos and Kenakin, 2002). Little is known about allosteric modulation of the GLP-1R, and few small non-peptide ligands acting allosterically at the GLP-1R have been reported. Recently, a number of small molecule agonists were identified by Novo-Nordisk, with the most potent of these, compound 2, increasing the affinity of GLP-1(7-36)NH_2, and also displaying intrinsic efficacy in cAMP accumulation assays (Knudsen et al., 2007). There is also a preliminary report that the naturally occurring flavonol, quercetin, may modulate GLP-1R-mediated calcium (Ca^{2+}) signalling by GLP-1(7-36)NH_2 (Schann et al., 2008, unpublished observations[2]) and evidence that the substituted cyclobutane, Boc5, may also act as an agonist of the receptor (Chen et al., 2007).

A major development in GPCR research is the recognition that different ligands can engender unique receptor conformations, giving rise to distinct signalling profiles. This concept of "ligand-induced stimulus bias" is particularly relevant to receptor systems that have multiple endogenous ligands, and is further complicated when allosteric ligands are considered.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Although allosteric drugs acting at the GLP-1R offer great promise as therapeutics, the consequences of allosteric modulation of the GLP-1R and whether such ligands promote or modify stimulus bias at the receptor have not been fully explored. Furthermore, the natural complexity of the GLP-1R system, encompassing numerous endogenous peptide agonists, provides potential for small molecule compounds to differentially modulate individual peptide responses, a behaviour termed "probe-dependence" (Kenakin, 2008). Consequently, we investigated the signalling and binding properties of these putative allosteric modulators in association with the physiologically relevant endogenous agonists of the GLP-1R, as well as the clinically used mimetic exendin-4. We demonstrate, for the first time, that compound 2 and quercetin each have distinct pharmacological profiles, exhibiting selective modulation of specific peptide agonists and engendering stimulus bias at the GLP-1R. These data have significant implications for how GLP-1 receptor targeted drugs are screened and developed, while the allosterically driven, agonist-selective, stimulus bias highlights the potential for distinct clinical efficacy depending on the properties of individual drugs.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM) and Fluo-4 AM were purchased from Invitrogen (Carlsbad, CA, USA). Foetal bovine serum (FBS) was purchased from Thermo Electron Corporation (Melbourne, VIC, Australia). AlphaScreen reagents, ^{125}I-exendin(9-39), 96-well UniFilter GF/C filter plates, 384-well Proxiplates and Microscint 40 scintillant were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). SureFire^{TM} extracellular signal-regulated kinases 1 and 2 (ERK1/2) reagents were obtained from TGR Biosciences (Adelaide, SA, Australia). The bicinchoninic acid (BCA)

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

protein assay kit was purchased from Thermo Scientific (Rockford, IL, USA). Compound 2 was generated according to the previously published method (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.

Cell culture. FlpIn Chinese hamster ovary (CHO) cells stably transfected with human GLP-1R (FlpInCHO-huGLP-1R) were generated using Gateway technology (Invitrogen) as previously described (May et al., 2007a). These cells expressed the human GLP-1R at a density of 123,500 ± 1,368 receptors/cell. Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, and incubated in a humidified environment at 37ºC in 5% CO_2. Untransfected CHO-FlpIn cells were used to control for receptor-independent effects. Additional control experiments were performed using COS-7 cells expressing the human CTa calcitonin receptor (Morfis et al., 2008).

Radioligand binding assay. Membrane preparations of FlpInCHO-huGLP-1R were prepared as described previously (Avlani et al., 2004). Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions, with bovine serum albumin (BSA) as standard. Competition binding assays were performed in 96-well plates using 20 μg of membrane expressing GLP-1R. Membranes were incubated in HEPES buffer (1 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 1% (w/v) BSA, pH 7.4) containing 0.5 nM ^{125}I-exendin(9-39) and increasing concentration of unlabelled ligand for 1 h. For interaction studies, competition of ^{125}I-exendin(9-39) binding by each orthosteric agonist was performed in the presence of increasing concentrations of either compound 2 or quercetin. For all experiments, non-specific binding was defined by 1 μM GLP-1(7-36)NH_2. Incubation was terminated by rapid filtration through Whatman GF/C

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

filters (pre-soaked in 0.03% (v/v) polyethylenimine (PEI) for a min of 2 h) using a 96-well TomTec harvester (Hamden, CT, USA). Filters were washed three times with 0.9% NaCl (w/v), 0.3% BSA (w/v) and allowed to dry before addition of 30 μL scintillant and determination of radioactivity by scintillation counting.

cAMP accumulation assay. FlpInCHO-huGLP-1R cells were seeded at a density of 50,000 cells/well into 96-well culture plates, and incubated overnight at 37ºC in 5% CO_2. Growth media was replaced with stimulation buffer (phenol-free DMEM containing 0.1% (w/v) BSA and 1 mM 3-Isobutyl-1-methylxanthine (IBMX)) and incubated for a further 1 h at 37ºC in 5% CO_2. Cells were stimulated with increasing concentrations of peptide ligand alone, allosteric ligand alone, or simultaneously with increasing concentrations of allosteric ligand and peptide, and incubated for 30 min at 37ºC in 5% CO_2. Selected additional experiments were also performed at 5 min and 10 min time points post ligand-stimulation to account for the potential for kinetic differences between different signalling assays to contribute to apparent stimulus bias. The reaction was terminated by rapid removal of the ligand-containing buffer and addition of 50 μL ice-cold 100% ethanol. Following ethanol evaporation, 75 μL of lysis buffer (0.1% (w/v) BSA, 0.3% (v/v) tween-20 and 5 mM HEPES, pH 7.4) was added and 10 μL of lysate was transferred to a 384-well proxiplate (PerkinElmer Life and Analytical Sciences). 5 μL acceptor bead mix (1.0% AlphaScreen cAMP acceptor beads diluted in lysis buffer) and 15 μL donor bead mix (0.3% AlphaScreen cAMP donor beads, 0.025% AlphaScreen cAMP biotinylated cAMP (133 Units/μL) diluted in lysis buffer, and preincubated for a minimum of 30 min) were added in reduced lighting conditions. Plates were incubated at room temperature overnight before measurement of the fluorescence using a Fusion-α plate reader (PerkinElmer Life and Analytical Sciences) with standard AlphaScreen settings. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

ERK1/2 phosphorylation assay. FlpInCHO-huGLP-1R cells were seeded at a density of 50,000 cells/well into 96-well culture plates, and receptor-mediated ERK1/2 phosphorylation was determined by using the AlphaScreen ERK SureFire protocol as previously described (May et al., 2007a). Initial ERK1/2 phosphorylation time course experiments were performed over 1 h to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by agonists. Cells were stimulated with peptide ligand and/or simultaneously with increasing concentrations of allosteric ligand for the time required to generate a maximal ERK1/2 phosphorylation response (10 min). Data were normalised to the maximal 3% FBS response, determined at 7 min (peak FBS response).

Intracellular Ca^{2+} mobilisation assay. FlpInCHO-huGLP-1R cells were seeded at a density of 50,000 cells/well into 96-well culture plates, and receptor-mediated intracellular Ca^{2+} mobilisation was determined as previously described (Werry et al., 2005). Due to their autofluorescence at high concentrations, increasing concentrations of allosteric modulators were added 30 min prior to addition of peptide agonist in the FlexStation (Molecular Devices; Palo Alto, CA, USA) to establish a basal fluorescence signal. 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 sec for 120 sec. Peak magnitude was calculated using 5-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration-response curves. Data were normalised to the maximal response elicited by 100 μM ATP.

Data analysis. All data obtained were analyzed in GraphPad Prism 5.02 (GraphPad Software Inc.; San Diego, CA, USA). Concentration response signalling data were analyzed using a three-parameter logistic equation as previously described (May et al., 2007a):

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - log[A])}} \qquad \text{(Equation 1)}$$

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

where 'Bottom' represents the y-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 x required to generate a response halfway between 'Top' and 'Bottom.' Similarly, this equation was used in inhibition binding, 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, while 'Top' defines radioligand binding in the absence of a competing ligand. In a similar manner, the IC_{50} value represents the molar concentration of x required to generate a response halfway between 'Top' and 'Bottom'.

Allosteric modulator inhibition binding data were subsequently analyzed according to an allosteric ternary complex model (May et al., 2007a) to determine ligand cooperativity. In this case, non depletion of ligands was assumed (Avlani et al., 2008):

$$Y = \frac{B_{max} \times [A]}{[A] + K_{App}} + NS \qquad \text{(Equation 2)}$$

where

$$K_{App} = \frac{K_A \times K_B}{\alpha \times [B] + K_B} \times \frac{1 + [I]/K_I + [B]/K_B + (\alpha' \times [I] \times [B])}{K_I \times K_B} \qquad \text{(Equation 3)}$$

where Y represents radioligand binding, B_{max} denotes maximal binding site density, and NS denotes the fraction of non-specific binding. [A] and K_A denote the concentration of radioligand and equilibrium dissociation constant for the radioligand, respectively. [B] and K_B denote the concentration of allosteric ligand and equilibrium dissociation constant for the allosteric ligand, respectively. [I] and K_I denote the concentration of peptide agonist used in competition with the radioligand and the equilibrium dissociation constant for the peptide agonist, respectively. α and α' represent cooperativity factors, which are measures of the magnitude and direction of the allosteric interaction between the modulator and the

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

radioligand, or the peptide agonist, respectively. Values of $\alpha > 1$ are indicative of a modulator-mediated increase in binding activity, while values of $0 < \alpha < 1$ are indicative of a modulator-mediated decrease in binding affinity.

cAMP interaction data were also analyzed with an operational model of allosterism:

$$Y = Basal + \frac{(E_{max} - Basal) \times (([A] \times (K_B + \alpha\beta \times [B]) + \tau[B] \times [B] \times EC_{50})^n)}{(([A] \times (K_B + \alpha\beta \times [B]) + \tau[B] \times [B] \times EC_{50})^n) \times (EC_{50}{}^n) \times (K_B + [B])^n)} \quad \text{(Equation 4)}$$

where E_{max} is the maximal possible response of the system (not the agonist), basal is the basal level of response in the absence of agonist, K_B denotes the functional equilibrium dissociation constant of the agonist (B), τ (tau) is an index of the coupling efficiency (or efficacy) of the agonist and is defined as RT/KE (where RT is the total concentration of receptors and KE is the concentration of agonist-receptor complex that yields half the maximum system response (E_{max})), and n is the slope of the transducer function that links occupancy to response. $\alpha\beta$ is the combined affinity-efficacy parameter that measures the magnitude and direction of the functional interaction between the modulator and peptide agonist.

To quantify signalling bias, which may be manifested either as selective affinity (K_A) and/or efficacy (τ) of an agonist for a given pathway, agonist concentration response curves data were analysed with an operational model (Gregory et al, 2010), but modified to directly estimate the ratio of τ/K_A , in a similar manner as described by Figueroa et al., (2009), for each pathway:

$$\frac{E_{max}\, x\, (\tau/K_A)^n\, x\, [B]^n}{[B]^n\, x\, (\tau/K_A)^n + (1 + [B]/K_B)^n} \quad \text{(Equation 5)}$$

where all other parameters are as defined for equation 4.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

All estimated parameters are expressed as logarithms (mean ± S.E.M.), and where relevant, statistical analysis performed by one-way ANOVA and Dunnetts post test using GraphPad Prism 5.02, and statistical significance accepted at $p < 0.05$.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Compound 2 and quercetin selectively modulate the binding affinity of antagonists and agonists of the GLP-1R. To establish the ability of the putative small molecule GLP-1R modulators, compound 2 and quercetin, to modify peptide binding affinity, equilibrium binding studies were performed between each of these modulators and the radio-labelled orthosteric antagonist ^{125}I-exendin(9-39). Analysis of the data using an allosteric ternary complex model revealed negative cooperativity between compound 2 and the antagonist, with the level of inhibition reaching a limit consistent with the predicted allosteric mode of inhibition (Table 1, Figure 1B). Quercetin also displayed weak negative cooperativity with ^{125}I-exendin(9-39) (Table 1, Figure 1B). Affinity estimates (pK_B) for compound 2 and quercetin were 7.58 ± 0.22 and 6.79 ± 0.41, respectively.

To establish the ability of compound 2 to modulate orthosteric agonist affinity, competition binding studies were performed with GLP-1(7-36)NH_2, GLP-1(7-37), GLP-1(1-36)NH_2, GLP-1(1-37), oxyntomodulin or exendin-4 in the absence or presence of increasing concentrations of compound 2 (Figure 2, Table 1, supporting information (SI) Figure S1) or quercetin (SI Figure S2, Table 1). These assays demonstrated that compound 2 displays 'probe dependence' (Leach et al., 2007), whereby the cooperativity between the orthosteric and allosteric binding sites is dependent on the orthosteric ligand present in the system. Compound 2 caused a concentration-dependent increase in the affinity of GLP-1(7-36)NH_2 (Figure 2A, Table 1) and GLP-1(7-37) (SI Figure S1A, Table 1). Compound 2 also displayed robust positive cooperativity for oxyntomodulin to a greater extent than either GLP-1(7-36)NH_2 or GLP-1(7-37) (Figure 2D, Table 1). In contrast, compound 2 minimally altered the binding of GLP-1(1-36)NH_2, GLP-1(1-37) and exendin-4, therefore displaying neutral cooperativity with these agonists (Figure 2B-C, SI Figure S1B, Table 1). No significant

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

modulation in affinity of any peptide agonist was observed in interaction binding studies with quercetin (SI Figure S2, Table 1).

Compound 2 but not quercetin selectively augments cAMP responses in an agonist specific manner at the GLP-1R. The GLP-1R preferentially couples to the Gαs pathway, activating adenylate cyclase and augmenting intracellular levels of cAMP, which in turn play a direct role in the secretion of insulin (Drucker et al., 1987). Compound 2 stimulated a robust increase in cAMP production displaying low potency partial agonism in the absence of an orthosteric ligand (Figure 3E, Table 2). In contrast, quercetin did not act as an agonist at the GLP-1R for cAMP (SI Figure S3G), while no response was seen in untransfected CHO-FlpIn cells for any of the ligands studied.

To investigate the potential for allosteric effects on peptide agonist-mediated cAMP signalling, interaction studies between the small molecule modulators and peptide ligands were performed. Co-addition of each of the peptides with compound 2 resulted in an observed elevation in cAMP at low concentrations of peptide agonist as a result of the intrinsic efficacy of compound 2 (Figure 3). Analysis of the interaction between compound 2 and each of the peptides with the allosteric operational model revealed combined affinity-efficacy (αβ) estimates (Table 2) that were consistent with the affinity cooperativity estimates from the binding studies (Table 1), suggesting that effects of compound 2 on peptide-mediated cAMP responses were driven principally by changes in affinity. Thus, where peptides exhibited neutral cooperativity with compound 2 in binding, as seen for exendin-4, GLP-1(1-36)NH_2 and GLP-1(1-37), the αβ estimates for compound 2 were not significantly different from 1 (Figure 3B–3C, SI Figure S4B, Table 2), whereas oxyntomodulin displayed greatest combined cooperativity with compound 2 (Figure 3D, Table 2), and GLP-1(7-36)NH_2 and GLP-1(7-37) only modest cooperativity (Figure 3A, SI Figure S4A, Table 2).

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

For the latter two peptides, this level of cooperativity was insufficient to elicit a significant change in potency, consistent with simulation of the interaction, based on the affinity cooperativity estimate, which predicts only a 0.06 log unit shift in potency at 10 μM compound 2 (SI Figure S5). Additional experiments following either 5 min or 10 min agonist stimulation revealed equivalent profiles of compound 2 interaction (not shown).

Quercetin did not influence the production of cAMP for any of the peptides used in this study (SI Figure S3). This is consistent with the binding data, where the presence of quercetin did not alter the binding profile of any peptide (SI Figure S2).

Quercetin but not compound 2 selectively modifies intracellular Ca^{2+} responses via the GLP-1R in an agonist specific manner. Given that allosteric ligands can bind simultaneously with orthosteric ligands and promote unique changes in receptor conformation, the resulting conformations may engender 'stimulus-bias' across different signalling pathways in the same cellular background (Urban et al., 2007). In addition to Gαs coupling, the GLP-1R couples to Gαq-proteins, resulting in mobilisation of intracellular Ca^{2+} (Hallbrink et al., 2001). To investigate whether compound 2 or quercetin could impose stimulus bias on the actions of the orthosteric peptide agonists we performed functional interaction assays for mobilisation of intracellular Ca^{2+}. Neither compound 2 nor quercetin displayed any intrinsic efficacy for the GLP-1R in this pathway (SI Figure S6E, Figure 4E). To assess the roles of these allosteric modulators, concentration-response curves were established for the peptide ligands in the presence and absence of increasing concentrations of compound 2 or quercetin. In contrast to the cAMP data, quercetin caused biphasic changes in peptide agonist potency and efficacy for GLP-1(7-36)NH_2, GLP-1(7-37) and exendin-4 (Figure 4A-C). Weak inhibition of peptide efficacy was observed between 10 nM and 1 μM quercetin, while augmentation of peptide efficacy was observed between 30 μM and 50 μM

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

quercetin. However, no significant modulation of efficacy was observed for oxyntomodulin, which itself is only a partial agonist for this signalling pathway (Figure 4D). In contrast to quercetin, compound 2 had no effect on peptide agonist-mediated Ca^{2+} responses (SI Figure S6). Loss of Ca^{2+} signal with the addition of 10 μM compound 2 was observed with all peptides studied, however, there was also a parallel reduction in the observed ATP response, indicating that this effect was an experimental artefact at this concentration of compound 2. In contrast, all ATP responses in the presence of increasing concentrations of quercetin remained robust and consistent, as did calcitonin-mediated Ca^{2+} signalling in CTa receptor expressing COS-7 cells, consistent with quercetin's effects on high affinity agonists being mediated at the level of the GLP-1 receptor. Neither GLP-1(1-36)NH_2 nor GLP-1(1-37) displayed any agonism in this signalling pathway at the tested concentration range (1 pM - 1 μM) in either the absence or presence of compound 2 or quercetin (SI Figure S6E, Figure 4E).

Neither compound 2 or quercetin modulate agonist–mediated ERK1/2 phosphorylation via the GLP-1R. ERK1/2 phosphorylation is often used as a general marker of convergent activation of multiple pathways, including G protein-independent signalling such as arrestin scaffolding (Lee et al., 2008). While we observed that both compound 2 and quercetin could engender stimulus bias for a subset of peptides, we failed to observe any modulation of agonist responses in ERK1/2 phosphorylation in the presence of compound 2 or quercetin (Figure 5, SI Figure S7). However, compound 2 displayed weak partial agonism for phosphorylation of ERK1/2 with a pEC_{50} of 5.76 ± 0.06 and a maximum of 51% of the GLP-1(7-36)NH_2 response (Figure 5G). In contrast, quercetin displayed no intrinsic agonism in any pathway tested.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Despite the increasing prevalence of "biologicals" (large molecular weight natural or modified peptides/proteins) for the clinical treatment of disease, low molecular weight, orally active compounds are still pursued as the idealised therapeutic drug. Traditionally, such drugs have been developed to mimic the properties of the natural ligand of the receptor by targeting the orthosteric binding site. While this approach has been broadly successful for many GPCRs, including adrenergic receptors and histamine receptors (Black, 1989), there are many cases where it has been problematic. Consequently, there has been increasing interest in the development of allosteric and/or bitopic drugs as a way of targeting these receptors (Valant et al., 2009). However, allosteric interactions are often complex with the potential for modulation of affinity and efficacy, independently or concomitantly, as well as potential for the allosteric ligand to exhibit its own intrinsic efficacy; efficacy modulation and intrinsic efficacy may also be manifested in a pathway dependent manner (May et al., 2007b). Furthermore, the nature of the allosteric interaction is "probe dependent" (May et al., 2007b). In many physiological systems this latter behaviour is largely irrelevant, as there is principally one endogenous ligand for the receptor. However, there are numerous examples where individual receptors can respond physiologically or pathophysiologically to multiple endogenous ligands, including the melanocortin receptors (α-MSH, ACTH, AgRP) (Tao, 2010), the parathyroid hormone (PTH) receptor 1 (PTH and PTHrP) (Gardella and Juppner, 2000), and GLP-1Rs. In this study we demonstrate that allosteric modulation of the GLP-1R is complex, with pathway dependent modulation of receptor response that is reliant upon both the peptide ligand and the allosteric modulator. Furthermore, it highlights that distinct peptide ligands can exhibit stimulus bias at the GLP-1R, and that allosteric modulators can impose further bias on this activity. Although some caution should be applied when interpreting data from a transfected cell background rather than a native GLP-1R expressing cell line,

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

collectively, these data stress the need for broad elucidation of mechanism of action when developing allosteric compounds.

The GLP-1R is pleiotropically coupled, eliciting signals via diverse pathways, including prominently, the formation of cAMP (Baggio and Drucker, 2007). The signalling downstream of GLP-1R activation leading to the release of insulin is well studied and is critically dependent upon the activation of Gαs and formation of cAMP with subsequent effects via protein kinase A (PKA) and exchange protein directly activated by cAMP (EPAC), including direct inhibition of K_{ATP} channels, cell depolarization and influx of extracellular Ca^{2+} (Baggio and Drucker, 2007). Nonetheless, there is also a role for mobilization of intracellular Ca^{2+} in augmentation of response (Baggio and Drucker, 2007), which is also manifested with Gαq-coupled receptors such as the muscarinic M3 acetylcholine receptor (AChR) (Gautam et al., 2008). Longer term effects on gene transcription and the preservation of β-cell mass involve multiple signalling pathways, both cAMP-dependent and -independent; the latter include activation of mitogen activated protein kinases (MAPKs) such as ERK1/2. However, for many therapeutically important effects, including the modulation of appetite, the underlying GLP-1R mediated signalling is not yet fully elucidated. Nonetheless, it is clear that the physiological response is a composite of the interplay between the various signalling pathways activated by individual ligands.

Evaluation of signalling across three pathways (cAMP production, elevation of intracellular Ca^{2+} and ERK1/2 phosphorylation) demonstrated, as expected, that all of the peptide agonists coupled the receptor most strongly to Gαs-mediated cAMP production. Each of the high affinity agonists, GLP-1(7-36)NH_2, GLP-1(7-37) and exendin-4 exhibited a similar profile of activation (Table 3), consistent with other functional and physiological studies with these peptides (Baggio and Drucker, 2007; Goke et al., 1993). In contrast, GLP-1(1-36)NH_2, GLP-1(1-37) and oxyntomodulin exhibited significant bias relative to GLP-1(7-36)NH_2.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Oxyntomodulin exhibited less preference for cAMP relative to ERK1/2, but similar preference for cAMP relative to Ca^{2+}, indicating that physiological responses to oxyntomodulin via the GLP-1R could differ from those elicited by GLP-1(7-36)NH_2 (Table 3). Oxyntomodulin, like GLP-1(7-36)NH_2 and GLP-1(7-37), is elevated postprandially, reaches higher plasma levels than that of GLP-1(1-36)NH_2 and GLP-1(1-37), and can reach very high levels in some conditions including celiac disease in children (Le Quellec et al., 1998). However, it is generally thought to play only a limited role therapeutically due to its lower affinity for the GLP-1R. While oxyntomodulin can bind to both the glucagon and GLP-1R, most of its physiological responses appear to be mediated via the GLP-1R, as demonstrated in GLP-1R knockout mice (Estall and Drucker, 2006). Oxyntomodulin, however, has a distinct physiological profile to that of GLP-1(7-36)NH_2 (Druce and Bloom, 2006; Maida et al., 2008), which is not fully consistent with a purely affinity driven reduction in signalling, but is consistent with the observations of stimulus bias in this study.

The flavonol quercetin lacked intrinsic activity, but selectively modulated intracellular Ca^{2+} responses for the high affinity agonists GLP-1(7-36)NH_2, GLP-1(7-37) and exendin-4, causing weak inhibition at low concentrations and significant augmentation of response at concentrations above 10 μM, but had no effect on oxyntomodulin response. Thus, quercetin imparts stimulus bias with respect to Ca^{2+} signalling, but in a peptide-agonist dependent manner, that latter consistent with a receptor-dependent mode of action. The selective bias for the high affinity peptides is consistent with the overlap in signalling pathway activation profile exhibited by the peptides; the latter suggesting that these peptides induce similar receptor conformations.

In contrast, analysis of the actions of compound 2 revealed intriguing differences in behaviour of peptide agonists and also in modulation of peptide agonist function. Compound 2 displayed intrinsic efficacy for cAMP accumulation and ERK1/2 phosphorylation, and

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

while more efficacious in the former, it demonstrated less bias between these pathways than the peptide agonists of the receptor (Table 3). A formal assessment of relative signalling via mobilisation of intracellular Ca^{2+} was not possible due to the non-specific loss of response seen at higher compound 2 concentrations in the CHO cell background. Such distinction in receptor activation by compound 2 versus peptide agonists is not surprising as it engages the receptor via different interactions and could be expected to engender a unique conformation(s). This type of behaviour has recently been observed at the M4 muscarinic ACh receptor, where the allosteric agonist LY2033298 activates the receptor by conformational shifts that are partially distinct from those used by orthosteric agonists (Nawaratne et al., 2010).

In binding assays, compound 2 displayed significant probe-dependence for modulation of affinity; negative cooperativity with the antagonist radioligand ^{125}I-exendin(9-39), strong positive cooperativity with oxyntomodulin, weaker positive cooperativity with GLP-1(7-36)NH_2 and GLP-1(7-37), and essentially neutral cooperativity with exendin-4 and full-length GLP-1 peptides. The differential modulation of affinity for peptide agonists versus antagonist is not necessarily surprising as compound 2 is an allosteric agonist and thus would be expected to favour binding to activated states of the receptor (Hall, 2000). However, the profile for modulation of peptide agonists reveals unanticipated effects that could translate into unexpected responses in preclinical and/or clinical evaluation of drug efficacy. In this regard, there were a number of important findings: the first was a markedly greater modulation of oxyntomodulin affinity relative to that for GLP-1(7-36)NH_2 and GLP-1(7-37), which manifested in the cAMP assay as significant augmentation of oxyntomodulin potency but minimal augmentation of truncated GLP-1 peptide signalling; this response could be expected to allow drugs with this type of profile to elicit actions via oxyntomodulin that would not normally be seen with circulating levels of the peptide (40-60 pM physiologically

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

and >500 pM in some pathophysiological states (Le Quellec et al., 1998)). A second important observation was that GLP-1(7-36)NH_2 and GLP-1(7-37) had a distinct interaction with compound 2 from that of exendin-4. As discussed above, exendin-4 and the truncated forms of GLP-1 are considered to be functionally equivalent, however, the positive cooperativity between compound 2 and truncated GLP-1 peptides versus neutral cooperativity with exendin-4 implies that the conformational state(s) adopted by exendin-4 is indeed different to that induced/occupied by GLP-1(7-36)NH_2 and GLP-1(7-37); this may lead to divergence in signalling outcomes between the endogenous peptides and exendin-4 may be detected if more broad analysis of signalling and/or receptor regulation is undertaken. Such differences in receptor interaction could underlie unexpected side effect profiles in susceptible populations.

Like quercetin, compound 2 only modulated peptide signalling from one of the three pathways assayed in the current study. While quercetin modulated GLP-1R-mediated intracellular Ca^{2+}, compound 2 modulated GLP-1R-mediated cAMP, suggesting that both modulators engender distinct forms of stimulus bias. Although efficacy cooperativity may be influenced by the propensity of an allosteric ligand to activate an individual pathway (Hall, 2000), simulation of compound 2 modulation of response based on affinity cooperativity, in the absence of intrinsic efficacy, predicts that there should be increased potency for each pathway, at least for oxyntomodulin. The absence of this effect indicates that compound 2 is generating true stimulus bias towards production of cAMP, relative to the other pathways measured. Since, mechanistically, allosteric interactions are due to conformational changes in the receptor as a result of co-occupancy of two ligands, and conformational differences are the driver for biased signalling, broad understanding of receptor signalling is critical to understanding the success/failure of allosteric drugs during clinical development. It also highlights the importance of determining the function of putative allosteric modulators in

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

both binding and signalling assays, and that discrimination of allosteric properties of small molecules depends on screening in multiple pathways, regardless of coupling strength. Similarly, for endogenous receptor systems that involve the interplay of multiple natural ligands, probe-dependence of allosteric drugs is a major factor that needs consideration during discovery and development, and indeed this could also extend to otherwise inert metabolic products of the ligands.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

References

Avlani V, May LT, Sexton PM, and Christopoulos A (2004). Application of a kinetic model to the apparently complex behavior of negative and positive allosteric modulators of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* **308:** 1062-1072.

Avlani VA, McLoughlin DJ, Sexton PM, and Christopoulos A (2008). The impact of orthosteric radioligand depletion on the quantification of allosteric modulator interactions. *J Pharmacol Exp Ther* **325:** 927-934.

Baggio LL, and Drucker DJ (2007). Biology of incretins: GLP-1 and GIP. *Gastroenterology* **132:** 2131-2157.

Black J (1989). Drugs from emasculated hormones: the principle of syntopic antagonism. *Science* **245:** 486-493.

Chen D, Liao J, Li N, Zhou C, Liu Q, Wang G, Zhang R, Zhang S, Lin L, Chen K, Xie X, Nan F, Young AA, Wang MW (2007). A nonpeptidic agonist of glucagon-like peptide 1 receptors with efficacy in diabetic db/db mice. *Proc Natl Acad Sci U S A.* **104:** 943-948.

Christopoulos A, and Kenakin T (2002). G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54:** 323-374.

Deacon CF, Johnsen AH, and Holst JJ (1995a). Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J Clin Endocrinol Metab* **80:** 952-957.

Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, and Holst JJ (1995b). Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly

degraded from the NH2-terminus in type II diabetic patients and in healthy subjects. *Diabetes* **44:** 1126-1131.

DeFronzo RA (1992). Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview. *Diabetologia* **35:** 389-397.

Druce MR, and Bloom SR (2006). Oxyntomodulin : a novel potential treatment for obesity. *Treat Endocrinol* **5:** 265-272.

Drucker DJ, Philippe J, Mojsov S, Chick WL, and Habener JF (1987). Glucagon-like peptide I stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proc Natl Acad Sci USA* **84:** 3434-3438.

Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, Ghatei MA, and Bloom SR (2001). Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. *Am J Physiol Endocrinol Metab* **281:** E155-161.

Elbrond B, Jakobsen G, Larsen S, Agerso H, Jensen LB, Rolan P, Sturis J, Hatorp V, and Zdravkovic M (2002). Pharmacokinetics, pharmacodynamics, safety, and tolerability of a single-dose of NN2211, a long-acting glucagon-like peptide 1 derivative, in healthy male subjects. *Diabetes Care* **25:** 1398-1404.

Estall JL, and Drucker DJ (2006). Glucagon and glucagon-like peptide receptors as drug targets. *Curr Pharm Des* **12:** 1731-1750.

Figueroa KW, Griffin MT, and Ehlert FJ (2009). Selectivity of agonists for the active state of M1 to M4 muscarinic receptor subtypes. *J Pharmacol Exp Ther* **328:** 331-342.

Gardella TJ, and Juppner H (2000). Interaction of PTH and PTHrP with their receptors. *Rev Endocr Metab Disord* **1:** 317-329.

Gautam D, Jeon J, Li JH, Han SJ, Hamdan FF, Cui Y, Lu H, Deng C, Gavrilova O, and Wess J (2008). Metabolic roles of the M3 muscarinic acetylcholine receptor studied with M3 receptor mutant mice: a review. *J Recept Signal Transduct Res* **28:** 93-108.

Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, and Goke B (1993). Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J Biol Chem* **268:** 19650-19655.

Gregory KJ, Hall NE, Tobin AB, Sexton PM, and Christopoulos A (2010). Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *J Biol Chem* **285:** 7459-7474.

Hall DA (2000). Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. *Mol Pharmacol* **58:** 1412-1423.

Hallbrink M, Holmqvist T, Olsson M, Ostenson CG, Efendic S, and Langel U (2001). Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/Galpha(o) activation. *Biochim Biophys Acta* **1546:** 79-86.

Kenakin T (2008). Functional Selectivity in GPCR Modulator Screening. *Comb Chem High Throughput Screen* **11:** 337-343.

Kieffer TJ, McIntosh CH, and Pederson RA (1995). Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* **136:** 3585-3596.

Knudsen LB, Kiel D, Teng M, Behrens C, Bhumralkar D, Kodra JT, Holst JJ, Jeppesen CB, Johnson MD, de Jong JC, Jorgensen AS, Kercher T, Kostrowicki J, Madsen P, Olesen PH, Petersen JS, Poulsen F, Sidelmann UG, Sturis J, Truesdale L, May J, and Lau J (2007). Small-molecule agonists for the glucagon-like peptide 1 receptor. *Proc Natl Acad Sci USA* **104:** 937-942.

Knudsen LB, Nielsen PF, Huusfeldt PO, Johansen NL, Madsen K, Pedersen FZ, Thogersen H, Wilken M, and Agerso H (2000). Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J Med Chem* **43:** 1664-1669.

Le Quellec A, Clapie M, Callamand P, Lehmann M, Kervran A, Bataille D, and Rieu D (1998). Circulating oxyntomodulin-like immunoreactivity in healthy children and children with celiac disease. *J Pediatr Gastroenterol Nutr* **27:** 513-518.

Leach K, Sexton PM, and Christopoulos A (2007). Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol Sci* **28:** 382-389.

Lee MH, El-Shewy HM, Luttrell DK, and Luttrell LM (2008). Role of beta-arrestin-mediated desensitization and signaling in the control of angiotensin AT1a receptor-stimulated transcription. *J Biol Chem* **283:** 2088-2097.

Maida A, Lovshin JA, Baggio LL, and Drucker DJ (2008). The glucagon-like peptide-1 receptor agonist oxyntomodulin enhances beta-cell function but does not inhibit gastric emptying in mice. *Endocrinology* **149:** 5670-5678.

May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, and Christopoulos A (2007a). Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. *Mol Pharmacol* **72:** 463-476.

May LT, Leach K, Sexton PM, and Christopoulos A (2007b). Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47:** 1-51.

Mitri J, and Hamdy O (2009). Diabetes medications and body weight. *Expert Opin Drug Saf* **8:** 573-584.

Morfis M, Tilakaratne N, Furness SG, Christopoulos G, Werry TD, Christopoulos A, Sexton PM (2008). Receptor activity-modifying proteins differentially modulate the G protein-coupling efficiency of amylin receptors. *Endocrinology* **149:** 5423-5431.

Nawaratne V, Leach K, Felder CC, Sexton PM, and Christopoulos A (2010). Structural determinants of allosteric agonism and modulation at the M4 muscarinic acetylcholine receptor: Identification of ligand-specific and global activation mechanisms. *J Biol Chem* (in press).

Olansky L (2010). Do incretin-based therapies cause acute pancreatitis? *J Diabetes Sci Technol* **4:** 228-229.

Tao YX (2010). The Melanocortin-4 Receptor: Physiology, Pharmacology, and Pathophysiology. *Endocr Rev* doi: 10.1210/er.2009-0037.

Teng M, Johnson MD, Thomas C, Kiel D, Lakis JN, Kercher T, Aytes S, Kostrowicki J, Bhumralkar D, Truesdale L, May J, Sidelman U, Kodra JT, Jorgensen AS, Olesen PH, de Jong JC, Madsen P, Behrens C, Pettersson I, Knudsen LB, Holst JJ, and Lau J (2007). Small molecule ago-allosteric modulators of the human glucagon-like peptide-1 (hGLP-1) receptor. *Bioorg Med Chem Lett* **17:** 5472-5478.

Toft-Nielsen MB, Damholt MB, Madsbad S, Hilsted LM, Hughes TE, Michelsen BK, and Holst JJ (2001). Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *J Clin Endocrinol Metab* **86:** 3717-3723.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, and Mailman RB (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320:** 1-13.

Valant C, Sexton PM, and Christopoulos A (2009). Orthosteric/allosteric bitopic ligands: going hybrid at GPCRs. *Mol Interv* **9:** 125-135.

Werry TD, Gregory KJ, Sexton PM, and Christopoulos A (2005). Characterization of serotonin 5-HT2C receptor signaling to extracellular signal-regulated kinases 1 and 2. *J Neurochem* **93:** 1603-1615.

Wild S, Roglic G, Green A, Sicree R, and King H (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* **27:** 1047-1053.

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes: PMS is a Principal Research Fellow and AC is a Senior Research Fellow of the National Health and Medical Research Council (NHMRC) of Australia. This work was funded in part by NHMRC Program [Grant 519461].

[1]CK, DW and JS contributed equally to this work.

[2]http://www.domaintherapeutics.com/dtect4.html#_Poster_2008

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Figure 1. Structure and binding interactions elicited by allosteric modulators of the human GLP-1R. (A) Structures of the human GLP-1R small molecule allosteric modulators used in this study. (B) Characterisation of the inhibition binding profiles of compound 2 and quercetin at the human GLP-1R in relation to the endogenous peptide agonist GLP-1(7-36)NH_2 using ^{125}I-exendin(9-39) as the radio-ligand and membranes prepared from FlpInCHO cells stably expressing the human GLP-1R. Data are normalised to total binding and are analyzed with an allosteric modulator titration curve as defined in (Equation 2) and (Equation 3), assuming non-depletion (compound 2 and quercetin) or a competitive inhibition model (GLP-1(7-36)NH_2). All values are mean ± S.E.M. of six to twelve independent experiments, conducted in duplicate. Non-specific binding, measured in the presence of 10^{-6}M exendin-4, ranged from 25-30% of total binding (dotted line in panel (B)). B, bound radioligand; B_o, binding in the absence of peptide ligand (total binding).

Figure 2. Characterisation of the inhibition binding of varying concentrations of compound 2 in the presence of (A) GLP-1(7-36)NH_2, (B) exendin-4, (C) GLP-1(1-36)NH_2 or (D) oxyntomodulin using membranes prepared from FlpInCHO cells stably expressing the human GLP-1R. Data are normalised to total radio-ligand binding and are analyzed with a one-site competition plus allosteric modulator curve as defined in (Equation 2) and (Equation 3), assuming non-depletion. All values are mean ± S.E.M. of four to six independent experiments, conducted in duplicate. Non-specific binding, measured in the presence of 10^{-6}M exendin-4, ranged from 25-30% of total binding. B, bound radioligand; B_o, binding in the absence of peptide ligand (total binding).

Molecular Pharmacology Fast Forward. Published on June 14, 2010 as DOI: 10.1124/mol.110.065664
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Characterisation of the interaction between compound 2 and (A) GLP-1(7-36)NH_2, (B) exendin-4, (C) GLP-1(1-36)NH_2 or (D) oxyntomodulin in a cAMP accumulation assay using FlpInCHO cells stably expressing the human GLP-1R. (E) compound 2 alone. Data are normalised to maximal peptide response, and analyzed with an operational model of allosterism as defined in (Equation 4). All values are mean ± S.E.M. of three to eight independent experiments, conducted in duplicate.

Figure 4. Characterisation of the interaction between quercetin and (A) GLP-1(7-36)NH_2, (B) exendin-4, (C) GLP-1(7-37) or (D) oxyntomodulin in an intracellular Ca^{2+} mobilisation assay using FlpInCHO cells stably expressing the human GLP-1R. (E) GLP-1(1-36)NH_2, GLP-1(1-37) or quercetin alone. Data are normalised to the maximal response elicited by 100 μM ATP and analyzed with a three-parameter logistic curve as defined in (Equation 1). All values are mean ± S.E.M. of four to eight independent experiments, conducted in duplicate. Statistical significance of changes in E_{max} in the presence of quercetin in comparison to the E_{max} of the peptide alone were determined by one-way ANOVA and Dunnetts post test, and indicated with an asterisk (*p<0.05).

Figure 5. Characterisation of the interaction between compound 2 and (A) GLP-1(7-36)NH_2, (B) exendin-4, (C) GLP-1(7-37) (D) GLP-1(1-36)NH_2, (E) oxyntomodulin or (F) GLP-1(1-37) in an ERK 1/2 phosphorylation assay using FlpInCHO cells stably expressing the human GLP-1R. (G) compound 2 alone. Data are normalised to maximal peptide response and analyzed with a three-parameter logistic curve as defined in (Equation 1). All values are mean ± S.E.M. of three independent experiments, conducted in duplicate.

Table 1. Differential effects of putative allosteric modulators used in this study on binding properties of peptide agonists of the human GLP-1R. Data was fit with a one-site competition plus allosteric modulator model as defined in (Equation 2) and (Equation 3), or 3 parameter logistic model to yield binding parameters.

	[a]pK_i or [b]pK_B	[c]$Log\alpha_{compound\ 2}$	$Log\alpha_{quercetin}$
GLP-1(7-36)NH_2	9.38 ± 0.07	0.74 ± 0.13 (5.49)	[d]N.D.
GLP-1(1-36)NH_2	6.47 ± 0.09	0.05 ± 0.09 (1.12)	N.D.
Exendin-4	9.63 ± 0.06	0.15 ± 0.18 (1.41)	N.D.
Oxyntomodulin	7.53 ± 0.11	1.11 ± 0.22 (12.88)	N.D.
GLP-1(7-37)	9.09 ± 0.04	0.47 ± 0.12 (2.95)	N.D.
GLP-1(1-37)	6.43 ± 0.26	0.10 ± 0.14 (1.26)	N.D.
^{125}I-Exendin(9-39)	-	-0.37 ± 0.04 (0.43)	-0.11 ± 0.02 (0.78)
Compound 2	7.58 ± 0.22	-	-
Quercetin	6.79 ± 0.41	-	-

[a]pK_i is the negative logarithm of the estimated affinity of the peptide agonist for the receptor.

[b]pK_B is the negative logarithm of the estimated affinity of the allosteric compounds.

[c]Logα is the logarithm of the of the cooperativity factor governing the allosteric interaction between the peptide ligand and modulator. Antilogarithms are shown in parentheses.

[d]Data unable to be experimentally defined or had incomplete curves are indicated by N.D. Data not calculated are represented by -. All values are mean ± S.E.M. from three to six independent experiments performed in duplicate.

Table 2. Differential effects of compound 2 on peptide agonists of the human GLP-1R in cAMP accumulation in FlpInCHO cells stably expressing the human GLP-1R. Data was analyzed with an operational model of allosterism as defined in (Equation 4).

	[a]pEC_{50}	[b]E_{max}	[c]Logαβ (compound 2)
GLP-1(7-36)NH_2	10.81 ± 0.03	100	0.69 ± 0.17 (4.90) *
GLP-1(1-36)NH_2	8.21 ± 0.03	96 ± 2	0.19 ± 0.19 (1.55)
Exendin-4	11.09 ± 0.03	99 ± 5	0.37 ± 0.16 (2.34)
Oxyntomodulin	8.95 ± 0.03	96 ± 4	1.40 ± 0.19 (25.12) *
GLP-1(7-37)	10.84 ± 0.04	98 ± 3	0.81 ± 0.19 (6.46) *
GLP-1(1-37)	7.86 ± 0.02	97 ± 1	0.06 ± 0.19 (1.15)
Compound 2	6.40 ± 0.07	83 ± 2	-
Quercetin	ND[d]	ND	-

[a]pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response.

[b]E_{max} represents the maximal response normalised to that of GLP-1(7-36)NH_2.

[c]Logαβ values represent the logarithm of the product of binding (α) and activation (β) cooperativity factors between compound 2 and the peptide agonists. Antilogarithms are shown in parentheses.

[d]Data unable to be experimentally defined or had incomplete curves are indicated by N.D. Data not calculated are represented by -. All values are mean ± S.E.M. of three to six independent experiments, conducted in duplicate. Data were analyzed with one-way ANOVA and Dunnetts post test, and statistical significance determined at $p < 0.05$ (*).

Table 3. Efficacy of agonists of the GLP-1R in FlpInCHO cells stably expressing the human GLP-1R.

	GLP-1(7-36)NH_2	GLP-1(7-37)	Exendin-4	GLP-1(1-36)NH_2	Oxyntomodulin	Compound 2
[a]Log τ/K_A						
cAMP	10.93 ± 0.03	10.89 ± 0.05	11.16 ± 0.03	8.29 ± 0.03	9.05 ± 0.03	6.36 ± 0.04
ERK1/2	9.09 ± 0.05	9.09 ± 0.06	9.02 ± 0.03	7.87 ± 0.12	8.05 ± 0.08	5.78 ± 0.11
Ca^{2+}	8.27 ± 0.32	7.93 ± 0.49	8.06 ± 0.51	ND[d]	5.75 ± 0.47	ND
[b]Log$[\tau/K_A]_{normalized}$						
cAMP	0	0.04 ± 0.05	-0.23 ± 0.04	2.64 ± 0.04	1.88 ± 0.04	4.57 ± 0.05
ERK1/2	0	0.00 ± 0.08	0.07 ± 0.06	1.22 ± 0.13	1.04 ± 0.09	3.31 ± 0.12
Ca^{2+}	0	0.34 ± 0.59	0.21 ± 0.60	ND	2.52 ± 0.57	ND
[c]Log[stimulus bias]						
[e]cAMP/ERK1/2	0	0.04 ± 0.28	-0.30 ± 0.25	1.42 ± 0.36 *	0.84 ± 0.31 *	1.26 ± 0.35 *
cAMP/Ca^{2+}	0	-0.30 ± 0.59	-0.44 ± 0.60	ND	-0.64 ± 0.57	ND
ERK1/2/Ca^{2+}	0	-0.34 ± 0.59	-0.14 ± 0.60	ND	-1.48 ± 0.58 *	ND

[a]tau (τ) is an index of the coupling efficiency (or efficacy) of the agonist and is defined as RT/KE (where RT is the total concentration of receptors and KE is the concentration of agonist-receptor complex that yields half the maximum system response (E_{max})). K_A is the affinity of the agonist. Ratios were determined using (Equation 5).

[b]Expressed relative to the Log tau/K_B values for GLP-1(7-36)NH_2, this is a measure of the relative strength of coupling of each individual agonist for an individual pathway, relative to that of the control agonist.

[c]Ratio of the efficacy of each agonist for the two pathways relative to the values for GLP-1(7-36)NH_2, this is a measure of the degree of stimulus bias exhibited by individual agonists across the pathways, relative to that of the control agonist.

[d]ND, not determined due to insufficient response.

[e]Log[stimulus bias] data were assessed for difference from "0" (null hypothesis, no bias relative to GLP-1(7-36)NH_2) through calculation of confidence intervals. Values that do not include "0" within the 95% confidence interval are indicated with (*). Data are mean ± S.E.M. of three to eight individual experiments.

Figure 1.

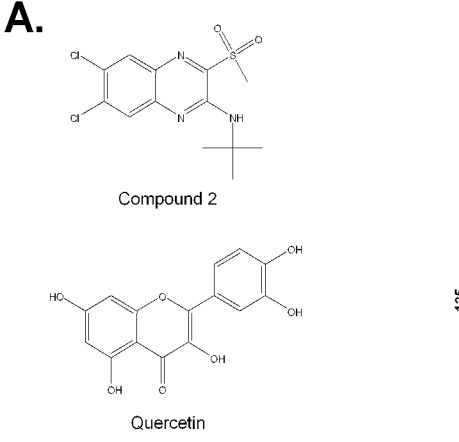
A.
Cl
Cl
N
N
S
O
O
NH
Compound 2
HO
O
OH
OH
OH
OH
O
Quercetin
B.
^{125}I-Exendin(9-39) Binding
(B/B$_o$ x 100)
120
100
80
60
40
20
0
GLP-1(7-36)NH$_2$
Compound 2
Quercetin
0
-12
-11
-10
-9
-8
-7
-6
-5
Log [Ligand] (M)

Figure 2.

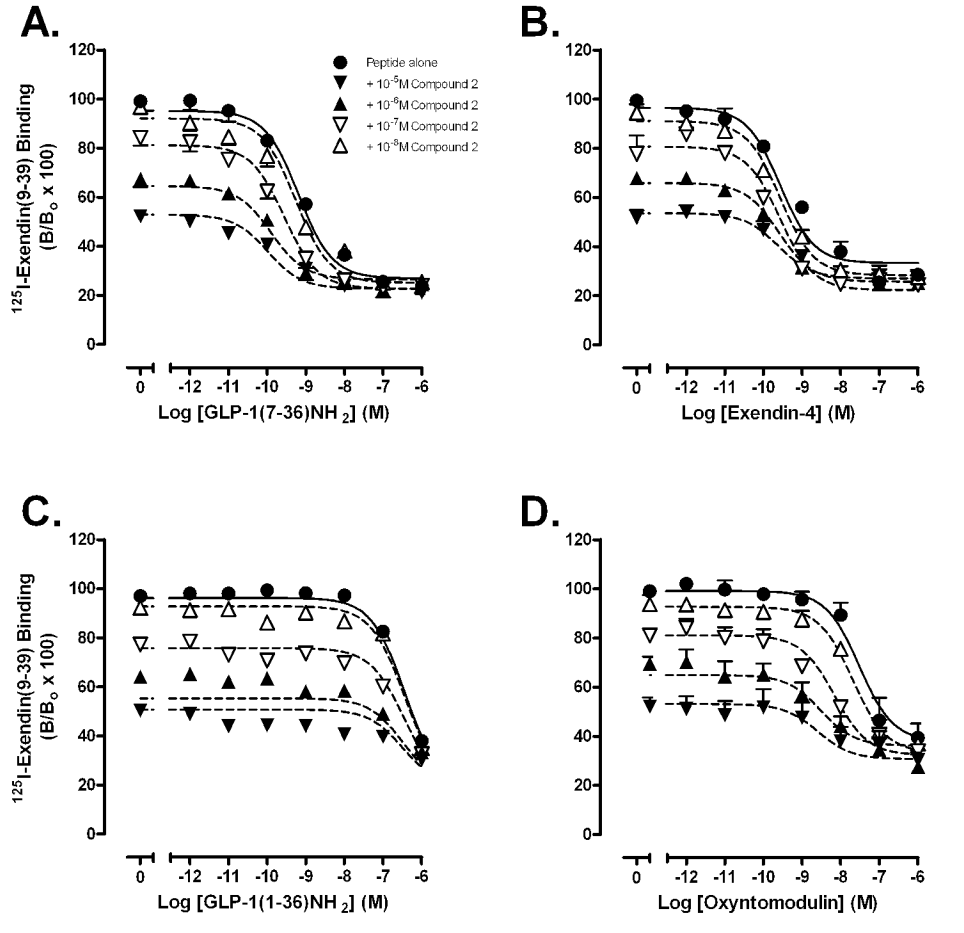
A.
B.
C.
D.
Peptide alone
+ 10-5M Compound 2
+ 10-6M Compound 2
+ 10-7M Compound 2
+ 10-8M Compound 2
125I-Exendin(9-39) Binding (B/Bo x 100)
Log [GLP-1(7-36)NH2] (M)
Log [Exendin-4] (M)
Log [GLP-1(1-36)NH2] (M)
Log [Oxyntomodulin] (M)
0
-12
-11
-10
-9
-8
-7
-6
0
20
40
60
80
100
120

Figure 3.

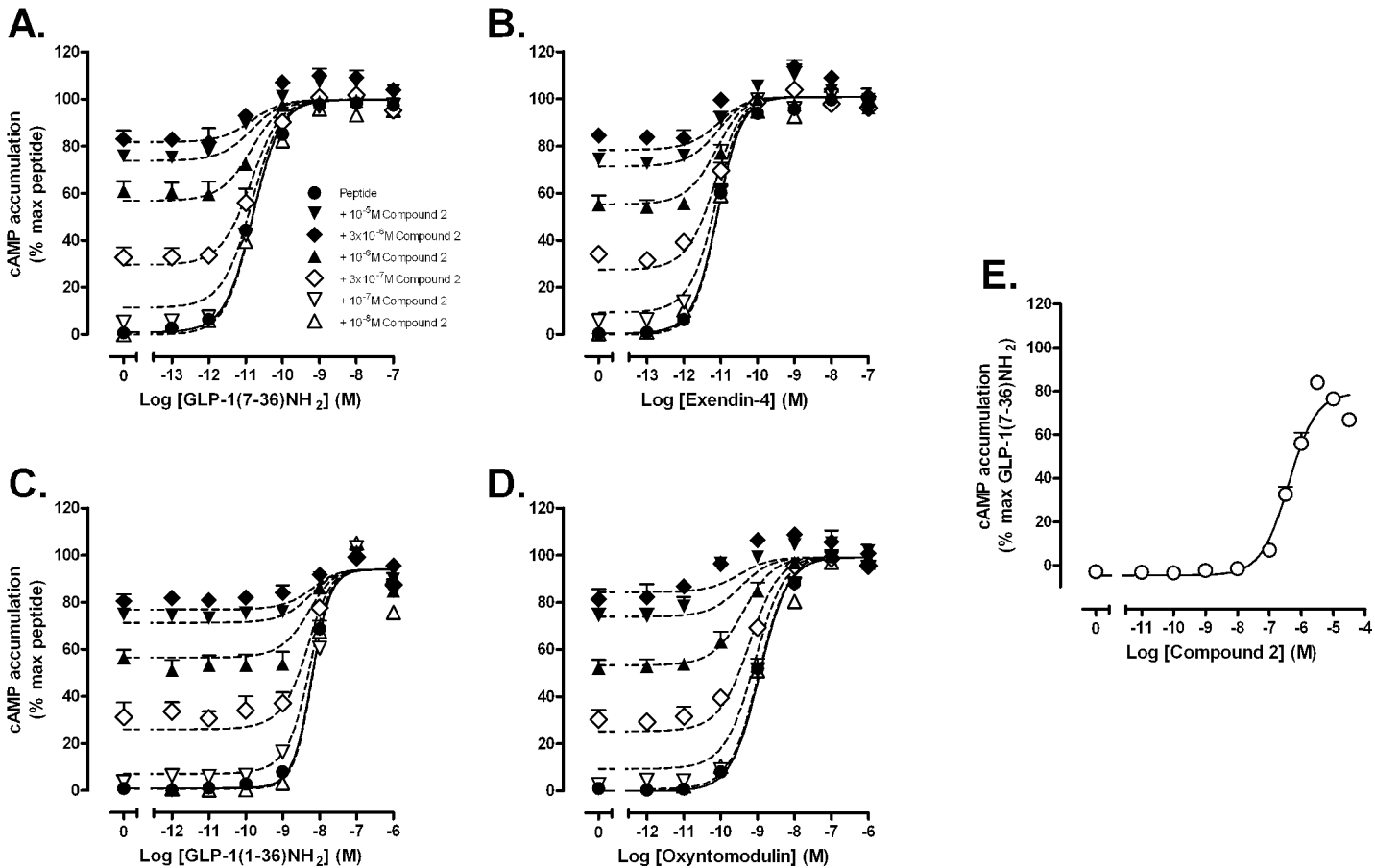
A.
cAMP accumulation (% max peptide)
Peptide
+ 10-5M Compound 2
+ 3x10-6M Compound 2
+ 10-6M Compound 2
+ 3x10-7M Compound 2
+ 10-7M Compound 2
+ 10-8M Compound 2
Log [GLP-1(7-36)NH2] (M)
B.
Log [Exendin-4] (M)
C.
Log [GLP-1(1-36)NH2] (M)
D.
Log [Oxyntomodulin] (M)
E.
cAMP accumulation (% max GLP-1(7-36)NH2)
Log [Compound 2] (M)

Figure 4.

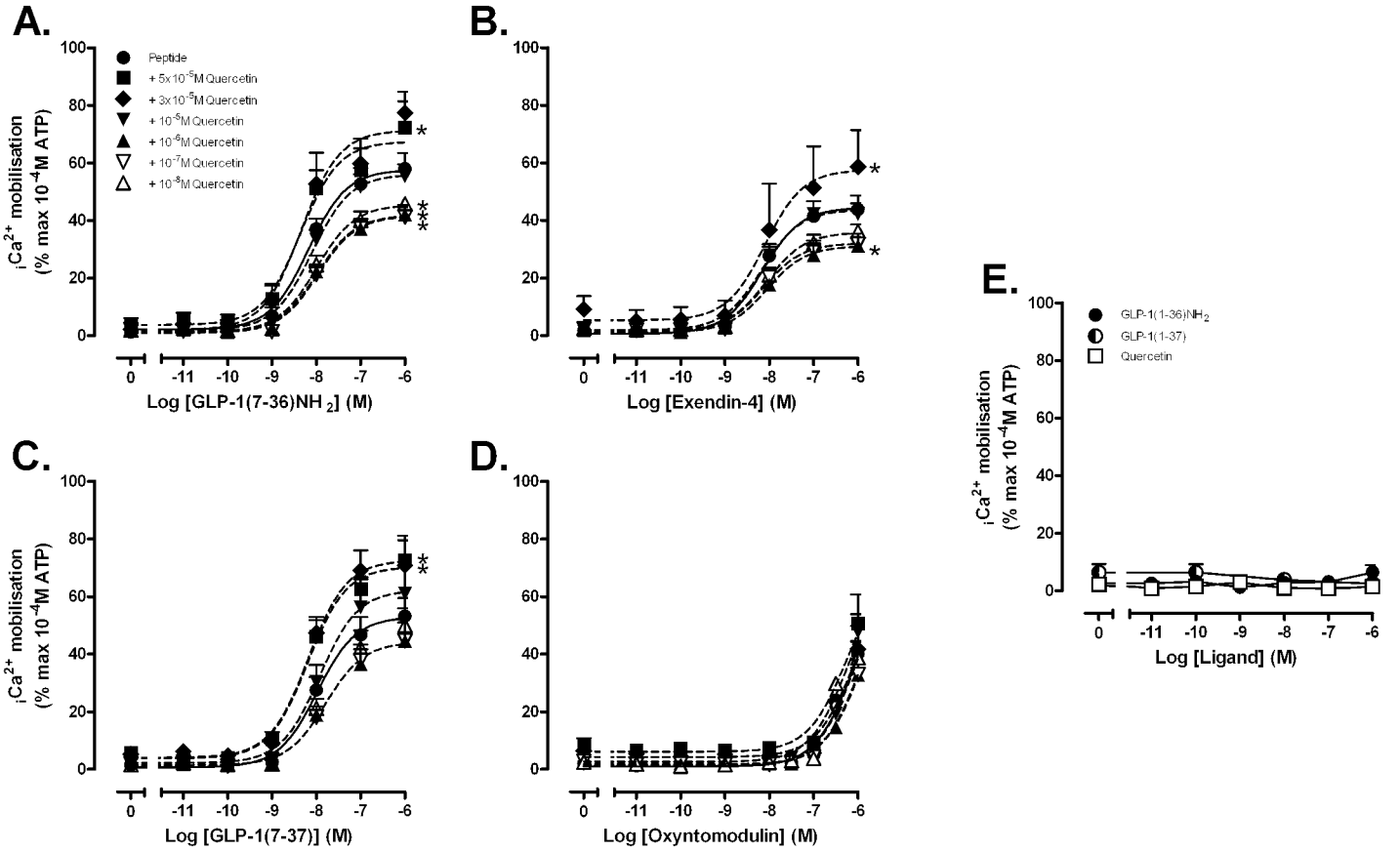
A.
B.
C.
D.
E.
Peptide
+ 5x10-5M Quercetin
+ 3x10-5M Quercetin
+ 10-5M Quercetin
+ 10-6M Quercetin
+ 10-7M Quercetin
+ 10-8M Quercetin
¡Ca2+ mobilisation (% max 10-4M ATP)
Log [GLP-1(7-36)NH2] (M)
Log [Exendin-4] (M)
Log [GLP-1(7-37)] (M)
Log [Oxyntomodulin] (M)
GLP-1(1-36)NH2
GLP-1(1-37)
Quercetin
Log [Ligand] (M)

Figure 5.