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Small Molecule Allosteric Modulation of the Glucagon-Like Peptide-1 Receptor Enhances the Insulinotropic Effect of Oxyntomodulin

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Running Title: Allosteric modulation of oxyntomodulin at the GLP-1 receptor

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Abbreviations: BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; BRET, bioluminescence resonance energy transfer; Ca^{2+}, calcium; CHO, Chinese hamster ovary; CNS, central nervous system; DPP-4, dipeptidyl peptidase-4; ERK1/2, extracellular signal-regulated kinases 1 and 2; $G\alpha_s$, α subunit of G protein; GCG, glucagon; GLP-1, glucagon-like peptide-1; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; HHB, hypotonic homogenization buffer; IVGTT, intravenous glucose tolerance test; OXM, oxyntomodulin; Rluc, Renilla luciferase.

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ABSTRACT. Identifying novel mechanisms to enhance glucagon-like peptide-1 (GLP-1) receptor signaling may enable nascent medicinal chemistry strategies aimed at developing new orally available therapeutic agents for the treatment of type 2 diabetes mellitus. Therefore, we tested the hypothesis that selectively modulating the low affinity GLP-1 receptor agonist, oxyntomodulin, would improve the insulin secretory properties of this naturally occurring hormone to provide a rationale for pursuing an unexplored therapeutic approach. Signal transduction and competition binding studies were used to investigate oxyntomodulin activity on the GLP-1 receptor in the presence of the small molecule GLP-1 receptor modulator, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine (BETP). *In vivo*, the intravenous glucose tolerance test characterized oxyntomodulin-induced insulin secretion in animals administered the small molecule. BETP increased oxyntomodulin binding affinity for the GLP-1 receptor and enhanced oxyntomodulin-mediated GLP-1 receptor signaling as measured by activation of the α subunit of heterotrimeric G protein and cAMP accumulation. In addition, oxyntomodulin-induced insulin secretion was enhanced in the presence of the compound. BETP was pharmacologically characterized to induce biased signaling by oxyntomodulin. These studies demonstrate that small molecules targeting the GLP-1 receptor can increase binding and receptor activation of the endogenous peptide oxyntomodulin. The biased signaling engendered by BETP suggests GLP-1 receptor mobilization of cAMP is the critical insulinotropic signaling event. Due to unique metabolic properties of oxyntomodulin, identifying molecules that enhance its activity should be pursued in order to assess the efficacy and safety of this novel mechanism.

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Introduction

The GLP-1 receptor mediates the predominant and best characterized physiologic actions of oxyntomodulin (glucagon-37), a peptide generated by tissue-specific, post-translational processing of proglucagon (Mojsov, Heinrich et al. 1986). This conclusion is drawn from a number of studies investigating the role of oxyntomodulin in glucose homeostasis, primarily as an insulinotropic hormone, and its effect on energy metabolism via signaling in the CNS. The most compelling data are from well-controlled experiments using GLP-1 receptor knockout mice; oxyntomodulin treatment improves glycemic control in both oral and intraperitoneal glucose tolerance tests in wild type but not in GLP-1 receptor null mice (Maida, Lovshin et al. 2008). These results are supported by *ex vivo* studies demonstrating that oxyntomodulin enhances glucose-stimulated insulin secretion in static cultures of isolated pancreatic islets from wild type but not GLP-1 receptor knockout mice (Maida, Lovshin et al. 2008). In the CNS, the metabolic dependence of GLP-1 receptor signaling for oxyntomodulin efficacy is shown in feeding studies where the anorectic action of intracerebroventricular injected oxyntomodulin is lost in GLP-1 receptor-deleted mice but preserved in animals lacking the glucagon receptor, another oxyntomodulin binding GPCR (Baggio, Huang et al. 2004). In addition to genetic ablation, studies using the peptide GLP-1 receptor antagonist, exendin-$4_{(9\text{-}39)}$, demonstrate that pharmacologic blockade of the GLP-1 receptor attenuates oxyntomodulin-induced insulin secretion from isolated islets and INS-1 832/3 cells (Maida, Lovshin et al. 2008), and it blunts oxyntomodulin-mediated inhibition of food intake (Dakin, Small et al. 2004).

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Demonstration that a functioning GLP-1 receptor is required for the major metabolic actions of oxyntomodulin is consistent with *in vitro* studies characterizing the ligand binding and receptor activation properties of oxyntomodulin on the GLP-1 receptor. Radioligand binding assays and cellular systems to measure cAMP accumulation show oxyntomodulin and GLP-1 (7-36)-NH_2/(7-37) are competitive ligands and full agonists of the GLP-1 receptor, although oxyntomodulin has lower binding affinity compared to GLP-1 (7-36)-NH_2/(7-37) (Fehmann, Jiang et al. 1994; Baggio, Huang et al. 2004). Oxyntomodulin is also a biased agonist at the GLP-1 receptor relative to GLP-1 (7-36)-NH_2/(7-37), exhibiting less preference towards cAMP signaling relative to phosphorylation of ERK1/2, indicating that physiologic responses to oxyntomodulin via the GLP-1 receptor could differ from those elicited by GLP-1 (7-36)-NH_2/(7-37) (Koole, Wootten et al. 2010).

While additional studies are needed to fully understand the physiologic significance of endogenous oxyntomodulin acting on the GLP-1 receptor, both GLP-1 (7-36)-NH_2/(7-37) and oxyntomodulin are derived from the same precursor protein and co-secreted upon meal ingestion (Mojsov, Heinrich et al. 1986; Le Quellec, Kervran et al. 1992). Importantly, differences in metabolic clearance of GLP-1 (7-36)-NH_2/(7-37) versus oxyntomodulin may enhance oxyntomodulin-mediated signaling at the GLP-1 receptor due to more rapid inactivation of GLP-1 (7-36)-NH_2/(7-37) by DPP-4 as it is a better DPP-4 substrate compared to oxyntomodulin (Zhu, Tamvakopoulos et al. 2003). The half-life of GLP-1 (7-36)-NH_2/(7-37) is 1-2 minutes (Siegel, Gallwitz et al. 1999), while half-life estimates for oxyntomodulin range from 6-12 minutes (Baldissera, Holst et al. 1988; Schjoldager, Baldissera et al. 1988; Kervran, Dubrasquet et al. 1990). Further,

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infusion studies in humans confirm the metabolic actions of oxyntomodulin (Cohen, Ellis et al. 2003), and new drug discovery approaches to develop long-acting analogs of oxyntomodulin are being pursued (Pocai, Carrington et al. 2009; Santoprete, Capito et al. 2011). While such molecules show initial success, these are peptide-based and require subcutaneous injection.

An alternate therapeutic approach is to enhance oxyntomodulin activation of the GLP-1 receptor with small molecular weight compounds that offer the potential to be developed as oral agents. We previously reported identification of a small molecule allosteric modulator of the GLP-1 receptor, BETP ("Compound B") (Sloop, Willard et al. 2010), and have also demonstrated proof-of-concept that pathway-specific signal transduction can be altered by low molecular weight compounds targeting the GLP-1 receptor (Koole, Wootten et al. 2010; Wootten, Savage et al. 2012). The studies herein were undertaken to explore whether BETP could modulate oxyntomodulin-induced activation of the GLP-1 receptor to enhance insulin secretion and characterize the influence of BETP on GLP-1 receptor signal transduction.

We show BETP is an affinity-driven, positive allosteric modulator for oxyntomodulin on the GLP-1 receptor *in vitro*. *In vivo*, BETP enhances the insulinotropic effect of oxyntomodulin in an intravenous glucose tolerance test model. We observe that BETP is a $G\alpha_s$/cAMP pathway biased allosteric modulator of oxyntomodulin, suggesting cAMP mobilization is the principle insulinotropic signal transduction pathway of the GLP-1 receptor.

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Materials and Methods

Ligands. BETP was synthesized at Eli Lilly and Company as previously reported (Sloop, Willard et al. 2010). GLP-1 (7-36)-NH_2 and oxyntomodulin were either purchased (Bachem, Torrance, CA, USA) or generated on solid support using an automated peptide synthesizer and Fmoc protocols. Following cleavage from the resin, crude peptides were purified on a C18 reverse-phase high-performance liquid chromatography column. After lyophilization, peptides were kept in powder form at -20°C and dissolved immediately prior to conducting experiments.

HEK293 and CHO Cellular Assays. HEK293 cells transiently expressing the human GLP-1 receptor at 80,000 receptors per cell or the human glucagon receptor were used for measurement of cAMP accumulation. Cells were grown at 37°C 5% CO_2 in Dulbecco's modified eagle's medium-SH30022 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 mM HEPES. Cells were transfected in suspension at 6.25 x 10^4 cells/ml contained in supplemented medium lacking antibiotics with a 6:1 FuGENE6 (Promega, Madison, WI, USA) transfection reagent to plasmid DNA ratio. Forty-eight hours following transfection, *i.e.* two hours prior to compound testing, cells were lifted, resuspended in 0.5% fetal bovine serum-supplemented (as above) Dulbecco's modified eagle's medium-31053 (Invitrogen, Carlsbad, CA, USA), and kept at 37°C. Transfected cells were seeded at a density of 2,500 cells/well into 96-well half-area, solid black microplates. Compounds, intermediately diluted in cell assay medium containing 0.1% bovine serum albumin fraction V and 3-Isobutyl-1-methylxanthine (250 µM final concentration), were added to the cells. Following a 20 minute incubation, cells were

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assayed for cAMP using homogenous time resolved fluorescence (Cisbio, Bedford, MA, USA) in 100 µl reactions. Fluorescence was measured according to the manufacturer's instructions using an Envision (Perkin Elmer, Boston, MA, USA). Data were analyzed by the ratio method and calibrated to external standards and expressed as percent cAMP compared to the reference peptide agonists. For CHO cellular assays, Flp-In CHO cells expressing the human GLP-1 receptor at a density of 120,000 receptors per cell were used; intracellular Ca^{2+} mobilization, ERK1/2 phosphorylation, and cAMP accumulation were measured as previously described (Wootten, Savage et al. 2012).

β-Arrestin Recruitment Assays. The pE5-frt-V5 pDestination vector (Invitrogen, Carlsbad, CA, USA) was modified to replace the V5 epitope with a modified Rluc8 in frame with the gateway cassette followed by an internal ribosome entry site and either β-arrestin1- or β-arrestin2-Venus fusions. The native encephalomyocarditis virus internal ribosome entry site was chosen as this is reported to produce 7-10 fold more protein from the second cistron (the β-arrestin-Venus acceptor fusion) than the first fulfilling the requirements for BRET of having the acceptor in excess of the donor (Bochkov and Palmenberg 2006). This construct was validated for use in arrestin translocation assays using the M_1 muscarinic acetylcholine receptor, demonstrating comparable ligand-induced response to experiments where donor:acceptor ratios have been optimized using transient transfection. The GLP-1 receptor cDNA without the stop codon was subcloned into the gateway cassette (using gateway technology) producing a GLP-1 receptor-Rluc8 fusion. Subsequently, Flp-In CHO cell lines stably expressing GLP-1 receptor-Rluc8 and either β-arrestin1- or β-arrestin2-Venus were generated using gateway technology. The fusion of Rluc8 to the C-terminus of the GLP-1 receptor

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did not alter its pharmacology as assessed in cAMP accumulation, phosphorlyated ERK1/2, and Ca^{2+} mobilization assays (data not shown). Cells were seeded in 96-well white culture plates at a density of 40,000 cells/well and cultured for 24 hours. Cells were rinsed once with Hank's balanced salt solution to remove traces of phenol red and incubated in fresh Hank's balanced salt solution for a further 15 minutes. The Rluc substrate coelenterazine-h was added to reach a final concentration of 5 µM. After a 5 minute incubation, the corresponding agonist was added and BRET readings were collected using a LumiSTAR Omega instrument that allows sequential integration of signals detected in the 465-505 and 515-555 nm windows using filters with the appropriate band pass. The BRET signal was calculated by subtracting the ratio of 515-555 nm emission over 465-505 nm emission for a vehicle-treated cell sample from the same ratio for the ligand-treated cell sample. In this calculation, the vehicle-treated cell sample represents background, and results are expressed as ligand-induced BRET. This eliminates the requirement for measuring a donor only control sample. Initial time course experiments were performed over 20 minutes to determine the time at which β-arrestin1 and β-arrestin2 recruitment was maximal for each ligand in the absence or presence of BETP. Co-addition of ligands was performed for interaction assays, and BRET signals were collected at this peak time point.

[^{35}S]GTPγS and [^{125}I]-exendin-4$_{(9-39)}$ Binding Assays. Membranes were prepared from HEK293 cells stably expressing the human GLP-1 receptor previously described (Sloop, Willard et al. 2010). Cells were resuspended at 5 ml/g cell paste in HHB: 25 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1x Complete inhibitors without EDTA (Roche, Indianapolis, IN, USA). The cell suspension was initially disrupted in the

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presence of 25 U/ml DNAse I with 20-25 strokes of a motorized, dounce homogenizer and teflon pestle and centrifuged at 1800g for 15 minutes to pellet intact cells. Low-speed pellets were again disrupted as above in HHB lacking enzyme and subsequently centrifuged. Low-speed supernatants were transferred to high-speed tubes, centrifuged 25000g for 30 minutes. High-speed pellets were resuspended in 2 ml HHB/g original cell paste and measured for protein content with bicinchoninic acid reagent (Pierce, Rockford, IL, USA) and colorimetric detection. Receptor activation was measured via [^{35}S]GTPγS binding to $G\alpha_s$ using an antibody capture scintillation proximity assay (DeLapp, McKinzie et al. 1999). Reactions contained 50 µg membrane in 20 mM HEPES pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 40 µg/ml saponin, 0.1% bovine serum albumin, and [^{35}S] 500 pM guanosine 5'-(γ-thio) triphosphate (Perkin Elmer, Boston, MA, USA). Peptide and allosteric modulator were diluted and co-treated to a final concentration of 1% dimethyl sulfoxide. Binding was induced for 30 minutes at ambient temperature prior to solubilizing with 0.2% NP40 detergent, 2 µg/ml rabbit anti-$G\alpha_s$/olf SC-383 polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA), and 1 mg anti-rabbit polyvinyltoluene beads (Perkin Elmer, Boston MA, USA). The detection mixtures were developed for 30 minutes, centrifuged at 80g for 10 minutes, and counted for 1 minute per well using a Trilux Microbeta (Perkin Elmer, Boston, MA, USA). The GLP-1 receptor binding assay using [^{125}I]-exendin-$4_{(9\text{-}39)}$ as the radioligand was performed as previously described (Wootten, Savage et al. 2012).

Animal Care and *In Vivo* Intravenous Glucose Tolerance Test. Animals were maintained in accordance with the Institutional Animal Use and Care Committee of Eli Lilly and Company and the National Institutes of Health "Guide for the Use and Care of

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Laboratory Animals". For animal treatment, compounds were solubilized in dosing solution containing 10% ethanol/solutol, 20% polyethylene glycol-400, and 70% phosphate buffered saline pH 7.4. The IVGTT studies were performed with male Wistar rats (Harlan, Indianapolis, IN, USA) and group housed at 3 per cage in polycarbonate cages with filter tops. Rats were maintained on a 12:12 hour light-dark cycle (lights on at 6:00 AM) at 21°C and received 2014 Teklad Global diet (Harlan, Indianapolis, IN, USA) and de-ionized water *ad libitum*. Rats were fasted overnight and anaesthetized with 60 mg/kg Nembutal® (Lundbeck, Deerfield, IL, USA) for the duration of the experiment. For glucose and compound administration, a catheter with a diameter of 0.84 mm (Braintree Scientific, Braintree, IL, USA) was inserted into the jugular vein. For rapid blood collection, a larger catheter with 1.02 mm diameter (Braintree Scientific, Braintree, IL, USA) was inserted into the carotid artery. Blood was collected for glucose and insulin levels at times 0, 2, 4, 6, 10, and 20 minutes after intravenous administration of the compound which was immediately followed by an intravenous glucose bolus of 0.5 g/kg. Plasma insulin was determined using an electrochemiluminescence assay (Meso Scale, Gaithersburg, MD, USA).

Data Analysis. Pharmacology data were fit using Prism 5.0 (GraphPad, La Jolla, CA, USA) using the four parameter logistic equation or an operation model of allosteric agonism. Allosteric modulator inhibition binding data were fitted with a one-site inhibition mass action curve (May, Leach et al. 2007) in order to determine ligand cooperativity. In this case, non depletion of ligands was assumed (Avlani, McLoughlin et al. 2008):

$$Y = \frac{B_{max} \times [A]}{[A] + K_{App}} + NS \qquad ((1)\ 1)$$

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where

$$K_{App} = \frac{K_A \times K_B}{\alpha \times [B] + K_B} \times \frac{1 + [I]/K_I + [B]/K_B + (\beta \times [I]\ X\ [B])}{K_I \times K_B} \quad (2)$$

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 β represent cooperativity factors, which are defined as the allosteric interaction of the modulator with the radioligand, and modulator with the peptide agonist, respectively. Values of α > 1 are indicative of an allosteric-mediated increase in binding activity, while values of 0 < α < 1 are indicative of an allosteric-mediated decrease in binding affinity. In cell signaling ligand interaction studies, data were fitted to the following two forms of an operational model of allosterism and agonism to derive functional estimates of modulator affinity and cooperativity (Leach, Sexton et al. 2007; Aurelio, Valant et al. 2009):

$$E = \frac{E_m\left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]K_A\right)^n}{\left([A]K_B + K_AK_B + [B]K_A + \alpha[A][B]\right)^n + \left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]K_A\right)^n} \quad (3)$$

$$E = \frac{E_m\left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]EC_{50}\right)^n}{EC_{50}{}^n\left(K_B + [B]\right)^n + \left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]EC_{50}\right)^n} \quad (4)$$

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where E_m is the maximum attainable system response for the pathway under investigation, [A] and [B] are the concentrations of orthosteric agonist and allosteric modulator/agonist, respectively, K_B is the dissociation constant of the allosteric modulator, EC_{50} is the concentration of orthosteric (full) agonist yielding 50% of the response between minimal and maximal receptor activation in the absence of allosteric ligand, n is a transducer slope factor linking occupancy to response, α is the binding cooperativity factor, β is an empirical scaling factor describing the allosteric effect of the modulator on orthosteric agonist signaling efficacy, respectively, and τ_A and τ_B are operational measure of the ligands' respective signaling efficacies that incorporate receptor expression levels and efficiency of stimulus-response coupling. Equation 3 was used in interaction studies performed between allosteric ligand (BETP) and a partial agonist (iCa^{2+}, β-arrestin1, and β-arrestin2), while equation 4 was used when BETP interacted with a full agonist (GTPγS, cAMP, and pERK1/2). This is because equation 2 is only valid in cases where the orthosteric agonist has high efficacy ($\tau >> 1$) such that K_A is >> [A].

Statistics. All data are represented as mean ± S.E.M. and were compared using analysis of variance followed by Dunnett's test. Repeated measures analysis of variance was used to assess the statistical significance between time courses. The null hypothesis was rejected at $p < 0.05$.

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Results

BETP Potentiates Oxyntomodulin-Induced Signaling

We previously demonstrated that BETP (**Figure 1A**) is a GLP-1 receptor ligand with micromolar intrinsic partial agonism in pancreatic islets and *in vivo* (Sloop, Willard et al. 2010). Moreover, BETP is an effective positive allosteric modulator of the naturally occurring, inactive GLP-1 metabolite GLP-1 (9-36)-NH_2 but shows little modulation of the active, circulating forms of GLP-1, GLP-1 (7-36)-NH_2/(7-37) (Wootten, Savage et al. 2012). Here, we hypothesized that BETP could be effective at potentiating endogenous GLP-1 receptor ligands with lower affinity than GLP-1 (7-36)-NH_2/(7-37), such as the comparatively low affinity, full agonist oxyntomodulin (Fehmann, Jiang et al. 1994; Baggio, Huang et al. 2004).

We quantified the ability of BETP to potentiate oxyntomodulin-induced cAMP accumulation in a heterologous system consisting of HEK293 cells expressing the human GLP-1 receptor (**Figure 1B**). In this system, GLP-1 (7-36)-NH_2 is a highly potent, full agonist (EC_{50} = 20 pM) whereas oxyntomodulin is a full agonist with 40-fold reduced potency (EC_{50} = 800 pM). In the presence of a submaximal concentration of BETP (4 μM), the potency of oxyntomodulin increases 10-fold (EC_{50} = 80 pM) while retaining full agonist efficacy. Consistent with our prior studies, BETP shows partial agonist activity with efficacy in the range of 10% of GLP-1 (7-36)-NH_2 (Wootten, Savage et al. 2012).

To assess whether the effect of BETP is specific and selective for the GLP-1 receptor, we examined oxyntomodulin-induced cAMP accumulation at the closely related glucagon receptor for which oxyntomodulin is hypothesized to be an endogenous ligand. We show that oxyntomodulin, a C-terminally extended form of

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glucagon, is a full agonist at the glucagon receptor with 20-fold lower potency (EC_{50} = 80 pM) than the canonical ligand glucagon (EC_{50} = 4 pM) (**Figure 1C**). In the presence of 4 μM BETP, the potencies and efficacies of both oxyntomodulin and glucagon at the glucagon receptor are unaltered, thus demonstrating BETP is a GLP-1 receptor selective positive allosteric modulator.

BETP Increases GLP-1 Receptor Binding of Oxyntomodulin

To determine the specific mechanism of BETP allosteric modulation of oxyntomodulin, we undertook radioligand binding studies to quantify the effect of BETP on oxyntomodulin affinity for the GLP-1 receptor. BETP dose-dependently increases the ability of oxyntomodulin to compete with [^{125}I]-exendin-$4_{(9\text{-}39)}$ for binding to the GLP-1 receptor in a whole cell binding assay (**Figure 2A**). We utilized an operational model of allosteric agonism to quantify cooperativity between BETP and oxyntomodulin (Leach, Sexton et al. 2007). BETP allosteric modulation of oxyntomodulin is affinity-driven with an α factor of 15, indicating that BETP increases the affinity of oxyntomodulin for the GLP-1 receptor by 15-fold (**Table 1**). To further confirm these data in a functional assay proximal to receptor activation, we used a $G\alpha_s$-specific GTPγS binding assay with membranes from GLP-1 receptor-expressing cells. In this system, basal signal is approximately 2,300 cpm while saturating GLP-1 (7-36)-NH_2 signal is approximately 12,000 cpm (**Supplemental Figure 1**). GLP-1 (7-36)-NH_2 and oxyntomodulin are both full agonists with potencies close to their binding affinities (Druce and Bloom 2006) (**Supplemental Figure 2**). BETP dose-dependently increases the potency of oxyntomodulin-stimulated G protein activation (**Figure 2B**), and at saturation, this is

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within 2-fold of the observed potency of GLP-1 (7-36)-NH_2 (**Supplemental Table 1**), indicating BETP is capable of potentiating the functional effects of oxyntomodulin to be on par with GLP-1 (7-36)-NH_2. BETP alone is a low potency partial agonist in the GTPγS binding assay entirely consistent with its pharmacological profile in cAMP accumulation assays (**Supplemental Figure 2**). We also examined the ability of BETP to potentiate GLP-1 (7-36)-NH_2 signaling at the GLP-1 receptor. We consistently observe that BETP does not enhance GLP-1 (7-36)-NH_2 potency or efficacy for activation of the GLP-1 receptor (**Supplemental Figure 3**).

BETP Enhances Oxyntomodulin-Induced Insulin Secretion

To test if BETP potentiation of oxyntomodulin could be observed in an intact physiological system, we performed an IVGTT in Wistar rats and measured insulin secretion as a functional endpoint (**Figure 3**). Here, infusion of GLP-1 (7-36)-NH_2 stimulates an acute insulinotropic response upon co-administration of a glucose bolus, consistent with the known biology of this peptide (Holst 2007). Similarly, oxyntomodulin dose-dependently induces insulin secretion and at saturating concentrations provides equivalent efficacy to that of GLP-1 (7-36)-NH_2, in-line with the proposition that oxyntomodulin and GLP-1 (7-36)-NH_2 are full agonists acting at the GLP-1 receptor to promote insulin secretion (Maida, Lovshin et al. 2008). BETP at the concentration used in these studies causes only a minimal degree of insulin secretion above vehicle alone. However, at subsaturating concentrations of oxyntomodulin, the insulinotropic effect of oxyntomodulin is markedly enhanced by co-administration of BETP (**Figure 3**). This effect is non-additive (the difference in insulin area under the curve, mean ± S.E.M. in

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ng/ml*min is as follows: BETP (5 mg/kg), 20 ± 6; oxyntomodulin (30 nmol/kg), 73 ± 8; BETP (5 mg/kg) + oxyntomodulin (30 nmol/kg), 179 ± 14) but rather synergistic, consistent with the hypothesis that BETP can increase the affinity of oxyntomodulin for the GLP-1 receptor and thereby increase the effectiveness of subsaturating doses of oxyntomodulin.

BETP Engenders Biased Signal Transduction

A potentially useful property of allosteric modulators is an ability to engender biased or functionally-selective signaling of orthosteric ligands. We previously showed that BETP engenders biased signaling by GLP-1 (9-36)-NH_2 with varying degrees of positive and negative cooperativity for cAMP accumulation, ERK1/2 activation, and Ca^{2+} mobilization pathways (Wootten, Savage et al. 2012). To quantify ligand bias induced by BETP potentiation of oxyntomodulin, we measured multiple signal transduction outputs in the same CHO cell line expressing the human GLP-1 receptor (**Figure 4**), and we fit data to an operational model of allosteric agonism to obtain quantitative descriptors of cooperativity and bias (**Table 1**). Affinity-driven positive cooperativity between BETP and oxyntomodulin is observed for the stimulation of cAMP accumulation (**Figure 4A, Table 1**), whereas neutral cooperativity for Ca^{2+} mobilization (**Figure 4B, Table 1**) and efficacy-driven negative cooperativity for ERK1/2 activation ($\beta = 0.03$) are observed (**Figure 4C, Table 1**). The other major reported pathway of GLP-1 receptor signaling is via β-arrestin (Jorgensen, Martini et al. 2005). We measured oxyntomodulin-induced β-arrestin1 recruitment using BRET (**Figure 4D**). Oxyntomodulin is a potent agonist of β-arrestin1 recruitment with potency and efficacy

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equivalent to GLP-1 (7-36)-NH_2 (**Supplemental Table 1**); likewise, BETP shows intrinsic partial agonism for β-arrestin1 recruitment (E_{MAX} = 45% of GLP-1 (7-36)-NH_2). Interaction experiments indicate BETP and oxyntomodulin are neutrally cooperative for β-arrestin1 recruitment but additive in nature (**Table 1**). An interesting finding is that BETP enhances the efficacy of GLP-1 receptor-mediated β-arrestin1 signaling, suggesting oxyntomodulin and GLP-1 (7-36)-NH_2 are only partial agonists in this system (**Supplemental Table 1**). Equivalent results are observed using the GLP-1 receptor and β-arrestin2 (**Supplemental Figure 4**). These data therefore indicate BETP allosteric modulation of oxyntomodulin in heterologous systems is strongly biased toward cAMP accumulation, neutral toward β-arrestin recruitment, and although not statistically significant, there is a trend towards negative bias for ERK1/2 activation **(Table 1)**.

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Discussion

Increasing the concentration of endogenous, active GLP-1 (7-36)-NH_2/(7-37) by orally administered DPP-4 inhibitors is a proven and effective therapeutic approach for improving glucose control in patients suffering from type 2 diabetes mellitus (Nauck, Meininger et al. 2007). However, treatment with injectable GLP-1 receptor agonist peptides provides additional therapeutic benefits over molecules of the DDP-4 inhibitor class as the GLP-1 analogs elicit larger %HbA1c reductions and often lead to significant weight loss (DPP-4 inhibitors are weight neutral) (Buse, Henry et al. 2004; DeFronzo, Ratner et al. 2005; Kendall, Riddle et al. 2005). The more profound metabolic efficacy shown by peptide-based molecules occurs as a result of delayed gastric emptying, reduced post-prandial hyperglucagonemia, and improved energy metabolism (DeFronzo, Okerson et al. 2008). These effects are GLP-1 receptor-dependent (Hansotia, Maida et al. 2007; Lamont, Li et al. 2012) and occur by achieving higher concentrations of circulating agonist. For example, therapeutic levels of the parenterally administered GLP-1 receptor agonist, exenatide, are greater than 8-fold the concentration of endogenous GLP-1 (7-36)-NH_2/(7-37) (DeFronzo, Okerson et al. 2008). Conversely, treatment with the DPP-4 inhibitor, sitagliptin, only raises the concentration of circulating GLP-1 (7-36)-NH_2/(7-37) by 2-fold (Herman, Stevens et al. 2005; DeFronzo, Okerson et al. 2008). These data indicate that higher exposure of GLP-1 receptor agonist improves treatment efficacy, and thus suggests there is additional therapeutic capacity by which orally available small molecules can enhance GLP-1 receptor activation beyond that achieved by DPP-4 inhibition.

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Recently, several groups have reported efforts to identify and explore development of nonpeptide, orally available GLP-1 receptor agonists or positive allosteric modulators (reviewed in Willard, Bueno et al. 2012). While discovery of surrogate agonists that utilize a receptor binding and activation mechanism similar to GLP-1 is likely difficult, we have reported that small molecules acting allosterically may be a more feasible approach (Koole, Wootten et al. 2010; Wootten, Simms et al. 2011). The data presented here explore the hypothesis that a GLP-1 receptor allosteric modulator can potentiate the activity of the endogenous hormone oxyntomodulin on the GLP-1 receptor, and thereby offer an additional small molecule approach to enhance GLP-1 receptor signaling. Targeting oxyntomodulin is an attractive therapeutic strategy for several reasons, including the post-prandial kinetic profile of oxyntomodulin secretion, its half-life, and its GLP-1 receptor binding properties. Similar to GLP-1 (7-36)-NH_2/(7-37), oxyntomodulin is released from endocrine L cells in the gut following meal ingestion (Le Quellec, Kervran et al. 1992), an important metabolic period when glucose-stimulated insulin secretion is needed to effectively reduce post-prandial hyperglycemia. From a treatment perspective, targeting oxyntomodulin action is advantageous because its half-life is approximately 6 times longer than that of GLP-1 (7-36)-NH_2/(7-37) (Baldissera, Holst et al. 1988; Schjoldager, Baldissera et al. 1988; Kervran, Dubrasquet et al. 1990). In addition, oxyntomodulin is a full GLP-1 receptor agonist, although its binding affinity is lower for the GLP-1 receptor compared to GLP-1 (7-36)-NH_2/(7-37) (Fehmann, Jiang et al. 1994; Baggio, Huang et al. 2004).

Importantly, this report shows proof-of-concept that a small molecule approach to potentiate oxyntomodulin activity on the GLP-1 receptor can be exploited

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pharmacologically to enhance insulin secretion. Mechanistically, BETP increases the binding affinity of oxyntomodulin for the GLP-1 receptor. The increase in GLP-1 receptor binding elicits a corresponding enhancement of GLP-1 receptor-stimulated activation of $G\alpha_s$ and increased formation of cAMP. Consistent with the established importance of cAMP signaling to potentiate glucose-stimulated insulin secretion, BETP enhances oxyntomodulin-induced insulin secretion in Wistar rats. Together, these results provide evidence to support pursing an "affinity-driven" medicinal chemistry strategy as a way to enhance the insulinotropic actions of oxyntomodulin. It is of note that BETP is a highly selective allosteric modulator as we only observe GLP-1 receptor-dependent action by this receptor when tested against a number of class B GPCRs (Sloop, Willard et al. 2010). Thus, the ability to potentiate the insulinotropic effects of oxyntomodulin without enhancing its actions on the glucagon receptor represents a desirable pharmacological characteristic for an anti-hyperglycemic agent.

Furthermore, in line with the effects on insulin secretion, an important finding of these studies is that at the oxyntomodulin-bound GLP-1 receptor, BETP induces biased signaling, selectively enhancing cAMP over Ca^{2+} mobilization, ERK1/2 phosphorylation, or β-arrestin recruitment. These data are consistent with our previous report showing functional selectivity of cAMP formation for a small molecule quinoxaline (often referred to as "Compound 2") for oxyntomodulin at the GLP-1 receptor (Koole, Wootten et al. 2010). The finding that two structurally distinct molecules show similar pharmacological effects on cAMP signaling is important for nascent efforts aimed at discovering novel potentiator chemotypes. While the *in vivo* effect of BETP on acute insulin secretion supports the hypothesis that modulating oxyntomodulin action improves glucose

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metabolism, unfortunately, physiochemical liabilities of BETP and Compound 2 (both are unstable in the presence of nucleophiles) limit longer term studies (Teng, Johnson et al. 2007; Willard, Bueno et al. 2012). For example, additional experiments are needed to explore the overall therapeutic consequence of preferentially enhancing cAMP signaling versus other pathways. Similarly, chronic studies are necessary to determine whether enhancing oxyntomodulin action on the GLP-1 receptor in the CNS improves energy metabolism leading to weight loss, a phenomenon shown for parenterally administered, long-acting oxyntomodulin analogs (Pocai, Carrington et al. 2009; Santoprete, Capito et al. 2011). A possible option for future long-term studies is to characterize the receptor binding properties and signal transduction capabilities of a recently disclosed quinoxaline analog. This compound is structurally similar to Compound 2, but it appears to have improved metabolic stability as data show mice orally dosed with the molecule display enhanced insulin secretion in an IVGTT (Kim, Kim et al. 2010; Moon, Yang et al. 2011). Thus, exploitation of biased GLP-1 receptor agonism using allosteric modulators to potentiate oxyntomodulin represents a novel theoretical approach for developing anti-diabetic agents. The use of focused medicinal chemistry and relevant pharmacological approaches appear to represent the clearest path to testing this hypothesis.

Although BETP and Compound 2 are unlikely to advance into clinical testing, pharmacological characterization of these molecules demonstrates several attractive features that may impact future screening and preclinical development schemes. Both compounds show partial intrinsic agonism on the GLP-1 receptor in the absence of peptide ligand, and neither is competitive with GLP-1 (7-36)-NH_2/(7-37) for receptor

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binding (Knudsen, Kiel et al. 2007; Sloop, Willard et al. 2010). Furthermore, we recently showed these molecules also activate the GLP-1 receptor by potentiating the DPP-4 cleaved, inactive metabolite GLP-1 (9-36)-NH_2, but not the parent agonist GLP-1 (7-36)-NH_2 (Wootten, Savage et al. 2012). These traits, combined with an ability to enhance oxyntomodulin activity on the GLP-1 receptor, represent an attractive activity profile for molecules that may provide an advance in the oral treatment of type 2 diabetes mellitus. Understanding the activity profile of these small molecule allosteric ligands may be key to drug discovery efforts, especially in systems like the GLP-1 receptor that contain multiple endogenous ligands. The physiologic need for the existence of multiple ligands acting at this receptor is still unclear, however, oxyntomodulin itself is a biased agonist, relative to GLP-1 (7-36)-NH_2 and therefore the two agonists may induce different physiological profiles. Also, the ability to selectively enhance the profile of one ligand over another, in addition to certain signaling pathways relative to others, may provide a therapeutic advantage by allowing fine tuning of receptor response; this could enhance the therapeutic effect while minimizing unwanted side effects. Further research is required to fully understand these concepts, and ascertaining the optimal signaling profile will require a library of allosteric ligands, each exhibiting different activity profiles. Future work should also explore the therapeutic potential of GLP-1 receptor allosteric modulators in treatment combinations with emerging small molecule incretin secretagogues that target fatty acid GPCRs located throughout the gastrointestinal tract. Exploiting the milleu of GLP-1 receptor ligands released by incretin secretagogues with GLP-1 receptor potentiators may enhance the emerging secretagogue approach.

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Authorship contributions

Participated in research design: *Alsina-Fernandez, Bokvist, Christopoulos, Ficorilli, Furness, Sexton, Sloop, Willard, Wootten*

Conducted experiments: *Farb, Ficorilli, Savage, Showalter, Wootten*

Contributed new reagents or analytic tools: *Alsina-Fernandez*

Performed data analysis: *Ficorilli, Savage, Showalter, Sloop, Willard, Wootten*

Wrote or contributed to writing of the manuscript: *Bokvist, Christopoulos, Sexton, Showalter, Sloop, Willard, Wootten*

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Footnote

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Figure Legends

Figure 1. Oxyntomodulin-stimulated GLP-1 receptor signaling is specifically enhanced by a small molecule GLP-1 receptor allosteric modulator. (A) The chemical structure of the low molecular weight GLP-1 receptor modulator, BETP, used in these studies. (B) Ligand-stimulated cAMP production is measured in HEK293 cells expressing the human GLP-1 receptor. Concentration response curves are depicted for oxyntomodulin (OXM) in the absence or presence of 4 μM BETP. Data are fit to the four parameter logistic equation and calculated EC_{50} and E_{MAX} values (% of maximal GLP-1 (7–36)-NH_2 effect) are as follows: GLP-1 (7-36)-NH_2, 21 pM, 100%; BETP, 740 nM, 7%; OXM, 770 pM, 98%; OXM + 4 μM BETP, 76 pM, 89%. (C) Ligand-stimulated cAMP production is measured in HEK293 cells expressing the glucagon (GCG) receptor. The potencies of GCG and OXM are determined from concentration response curves in the absence or presence of 4 μM BETP. Data are fit to the four parameter logistic equation. EC_{50} and E_{MAX} values (% of maximal OXM effect) are as follows: GCG, 4 pM, 101%; GCG + 4 μM BETP, 4 pM, 94%; OXM, 80 pM, 100%; OXM + 4 μM BETP, 79 pM, 91%. All data in (B) and (C) are mean ± S.E.M. of three to five independent experiments conducted in duplicate.

Figure 2. BETP increases the binding affinity of oxyntomodulin for the GLP-1 receptor and enhances activation of the $G\alpha_s$ heterotrimeric G protein. (A) Positive binding cooperativity of BETP and oxyntomodulin (OXM) is demonstrated in competition binding studies using GLP-1 receptor-expressing membranes. The potency of OXM to

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displace specific binding of [^{125}I]-exendin-4$_{(9-39)}$ is measured in the presence of fixed concentrations of BETP. (B) OXM-mediated [^{35}S]GTPγS binding to endogenous Gα_s protein in GLP-1 receptor-expressing membranes is determined by antibody capture scintillation proximity. The potency of OXM for G protein activation is measured at fixed concentrations of BETP. Data from (A) and (B) represent the mean ± S.E.M. from three experiments conducted in duplicate. Data are fit to the operational model of allosteric agonism; resultant calculated parameters are reported in **Table 1**.

Figure 3. Oxyntomodulin-stimulated insulin secretion is enhanced by BETP *in vivo*. (A) Time course of plasma insulin concentrations in fasted, anaesthetized Wistar rats treated with either vehicle, GLP-1 (7-36)-NH_2 (3 nmol/kg), OXM (30 nmol/kg), BETP (5 mg/kg), or OXM (30 nmol/kg) plus BETP (5 mg/kg) immediately prior to intravenous administration of a glucose bolus (0.5 g/kg). (B) Integrated area under the curve calculations for insulin secretion measurements. Data include additional 0.3 and 3 nmol/kg OXM treatment conditions with and without BETP (5 mg/kg). Results are expressed as mean ± S.E.M. Analysis of variance is used to assess statistical significance: * versus vehicle $p < 0.05$; # OXM alone condition versus OXM + BETP treatment conditions $p < 0.05$. Data represent three experiments.

Figure 4. BETP induces cAMP biased signaling at the oxyntomodulin-bound GLP-1 receptor. The dose-dependent effects of BETP on oxyntomodulin (OXM)-mediated activation of (A) cAMP accumulation, (B) intracellular Ca^{2+} mobilization, (C) ERK1/2 phosphorylation, and (D) β-arrestin recruitment are quantified in Flp-In CHO cells

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expressing the GLP-1 receptor. Data are fit to the operational model of allosteric agonism to quantify biased signaling; resultant calculated parameters are reported in **Table 1**.

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Table 1

Allosteric model parameters describing the cooperativity for the interaction between BETP and oxyntomodulin. α is the cooperativity factor that defines the fold change in affinity of oxyntomodulin by BETP and is calculated using a one-site competition plus allosteric modulator curve as defined in equations 1 and 2. αβ is the cooperativity factor that defines the fold change in receptor signaling by the BETP and is a composite factor describing the combined affinity (α) and efficacy (β) modulation by the allosteric ligand. This is calculated using an operational model of agonism as defined in equations 3 and 4. pIC_{50}, pEC_{50}, and Emax values for these data sets are presented in **Table S1**.[a] The pK_B value (the negative logarithm of the affinity) for BETP derived from application of the operational model of allosterism is 5.01 ± 0.09.

Assay Measurement	***Log α (α)***	***Log αβ (αβ)***	***Log β (β) (Log αβ – Log α)***
Binding (Affinity)	***1.16 ± 0.10 (14.6)[a]****	***-***	***-***
GTPγS	***-***	***1.11 ± 0.05 (12.9)****	***-0.05 ± 0.11 (0.89)***
cAMP	***-***	***1.10 ± 0.08 (12.6)****	***-0.06 ± 0.13 (0.87)***
pERK1/2	***-***	***-0.44 ± 0.19 (0.36)***	***-1.60 ± 0.21 (0.03)****
Ca^{2+}	***-***	***0.23 ± 0.11 (1.70)***	***-0.36 ± 0.39 (0.44)***
β-Arr1	***-***	***0.40 ± 0.08 (2.51)***	***-0.76 ± 0.13 (0.17)***
β-Arr2	***-***	***0.54 ± 0.19 (3.47)***	***-0.62 ± 0.21 (0.24)***

* Statistically significant at $p < 0.05$.

Figure 1

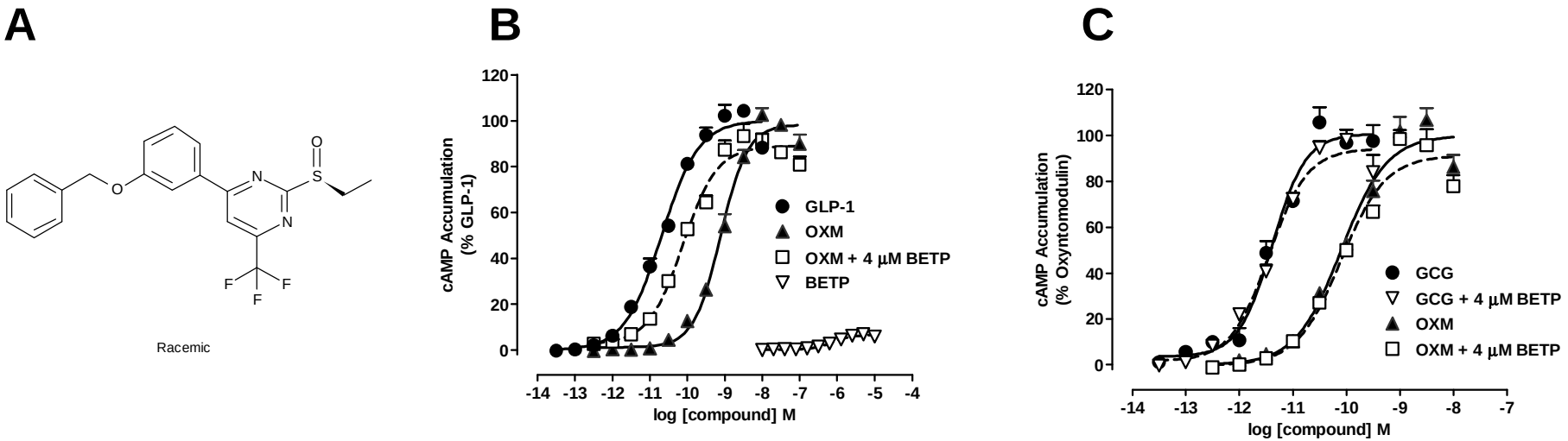


Figure 2

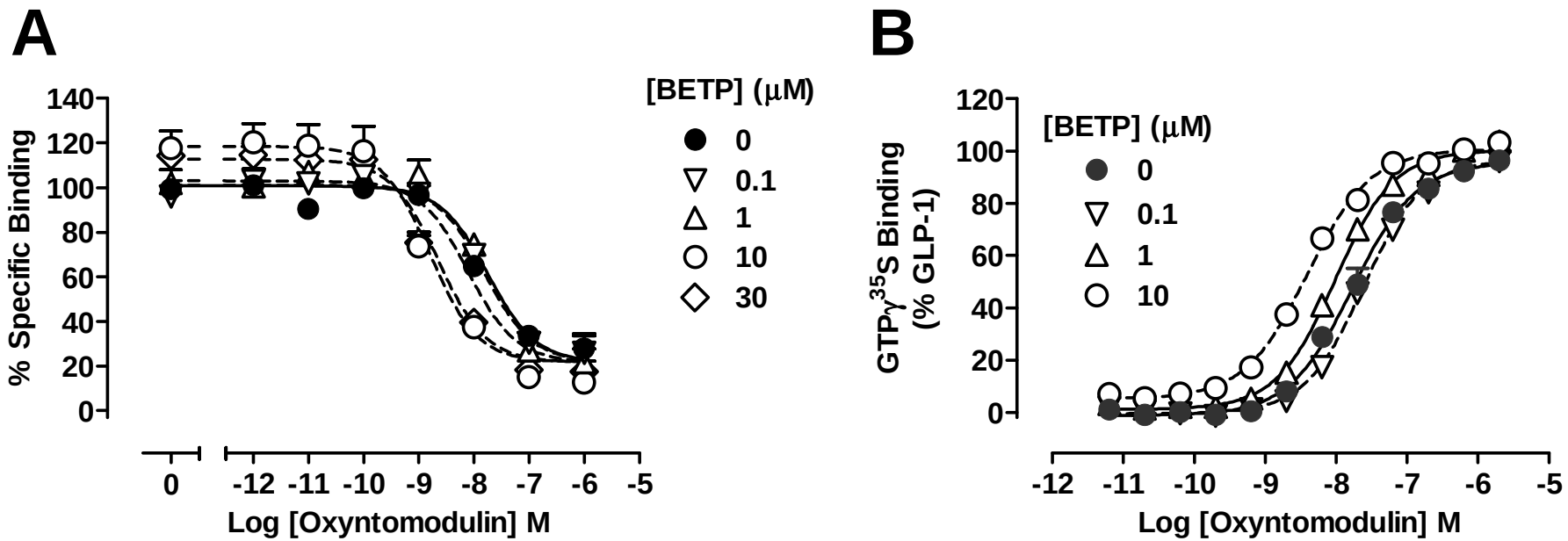


Figure 3

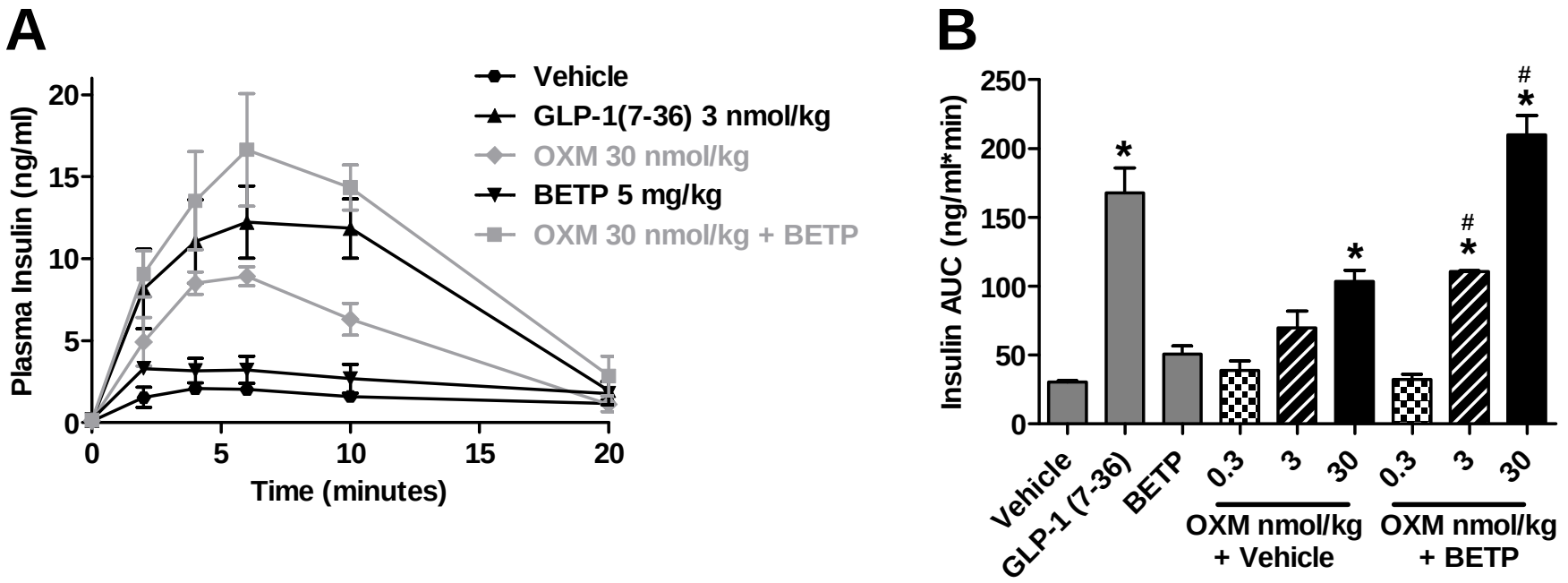
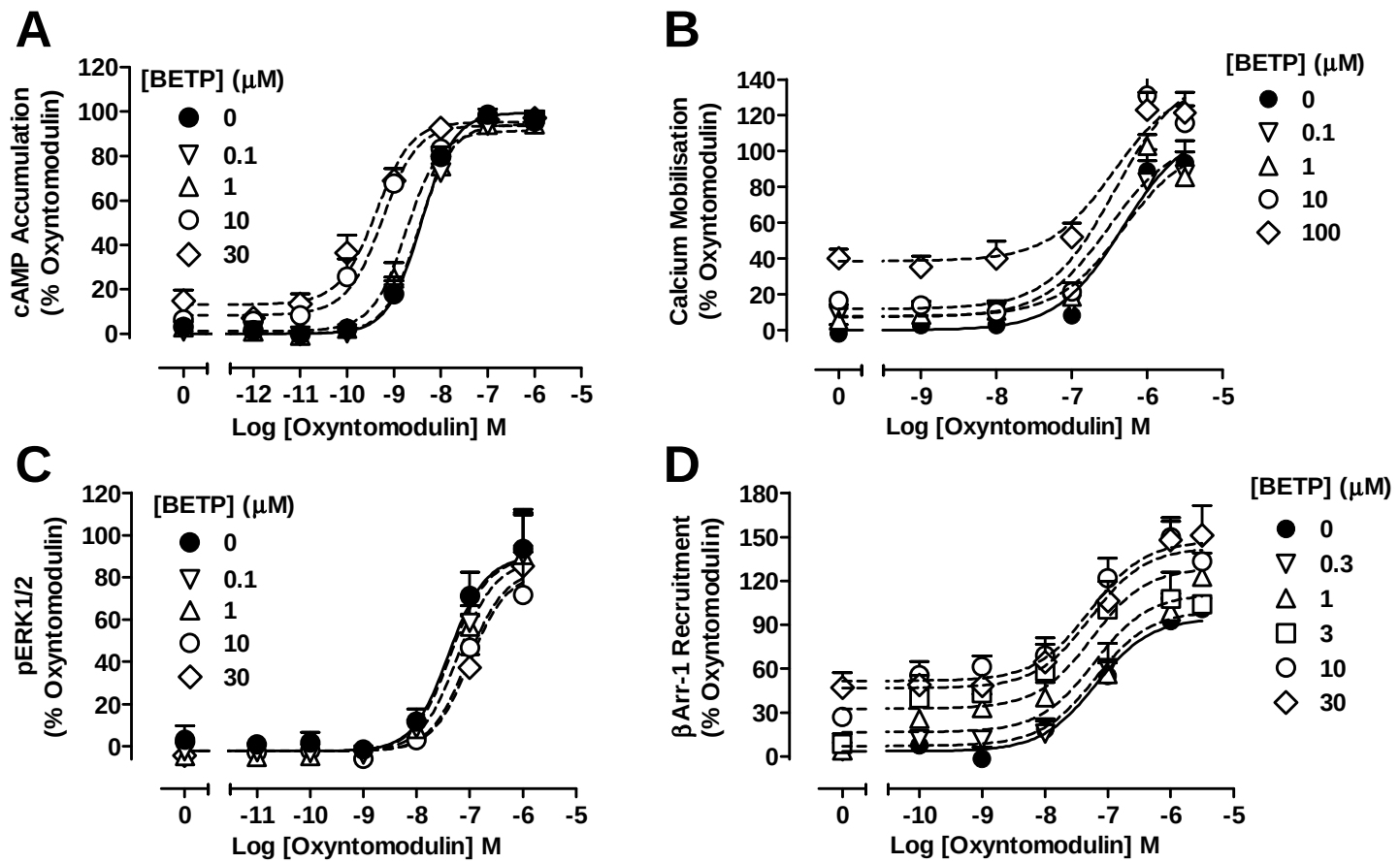


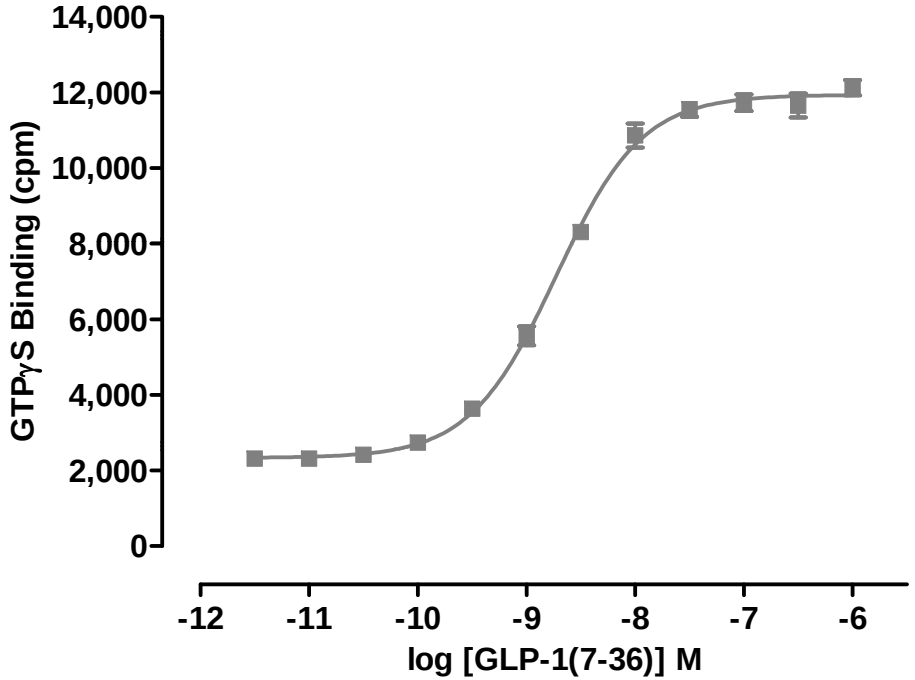
Figure 4



Molecular Pharmacology

Small Molecule Allosteric Modulation of the Glucagon-Like Peptide-1 Receptor Enhances the Insulinotropic Effect of Oxyntomodulin.

Francis S. Willard, Denise Wootten, Aaron D. Showalter, Emilia E. Savage, James Ficorilli, Thomas B. Farb, Krister Bokvist, Jorge Alsina-Fernandez, Sebastian G. B. Furness, Arthur Christopoulos, Patrick M. Sexton, and Kyle W. Sloop



Supplementary Figure 1

Ligand mediated [^{35}S]GTPγS binding to endogenous $G\alpha_s$ protein in GLP-1 receptor expressing membranes is measured by antibody capture scintillation proximity. Saturating GLP-1 concentrations induced a 5.2-fold increase in GTPγS binding as measured in raw cpm values. Data are fit to the four parameter logistic equation. EC_{50} of GLP-1 (7-36)NH_2 was 1.9 nM. Data are the mean ± S.E.M. of a single experiment conducted in triplicate and are representative of three independent experiments.

Molecular Pharmacology

Small Molecule Allosteric Modulation of the Glucagon-Like Peptide-1 Receptor Enhances the Insulinotropic Effect of Oxyntomodulin.

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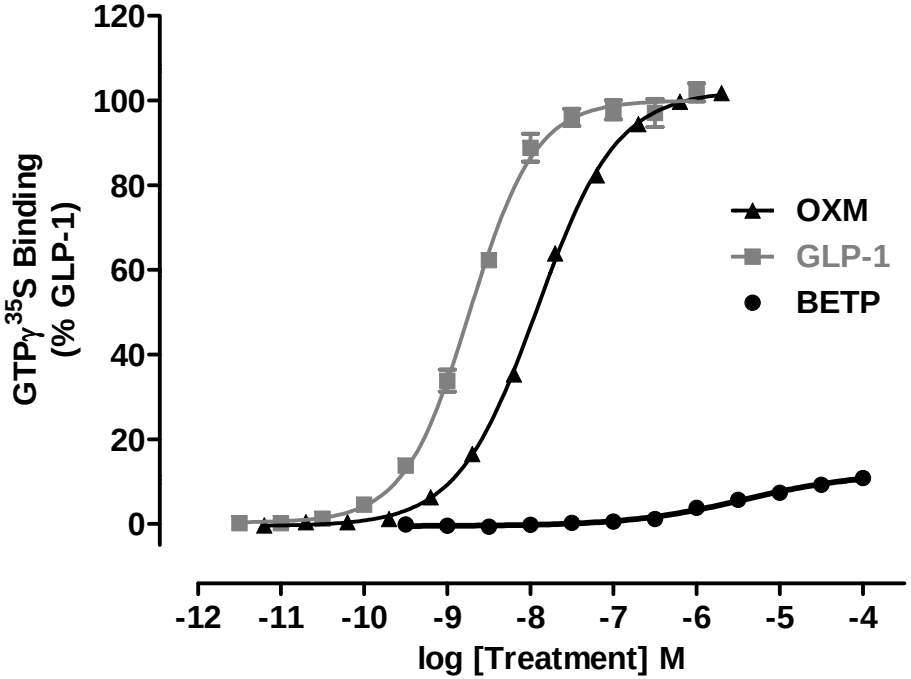

Supplementary Figure 2

Intrinsic efficacy of BETP, oxyntomodulin (OXM), and GLP-1 (7–36)NH_2 for G protein activation. Ligand mediated [^{35}S]GTPγS binding to endogenous Gα_s protein in GLP-1 receptor expressing membranes is measured by antibody capture scintillation proximity. Data are normalized to control values measured from saturating GLP-1 (7–36)NH_2 concentrations. Data are fit to the four parameter logistic equation. EC_{50} and E_{MAX} values (% of maximal GLP-1 (7–36)NH_2 effect): BETP, 3.7 µM, 12%; OXM, 12 nM, 103%; GLP-1 (7–36)NH_2, 1.9 nM, 100 %. Data are the mean ± S.E.M. of a single experiment conducted in triplicate and are representative of three independent experiments.

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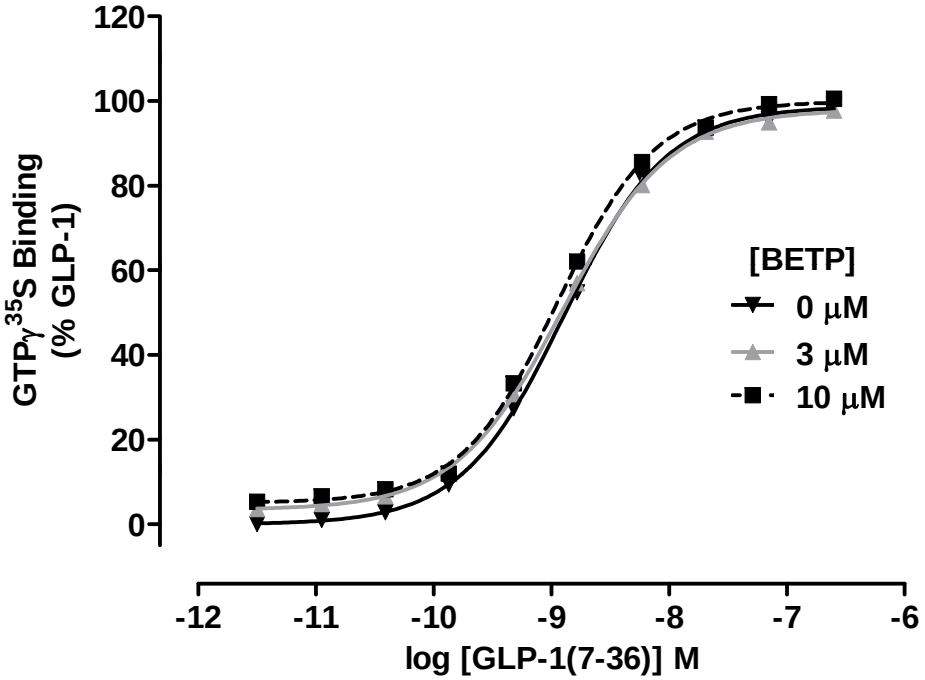

Supplementary Figure 3

The effect of BETP on GLP-1 (7–36)NH_2 mediated G protein activation. Ligand mediated [^{35}S]GTPγS binding to endogenous $G\alpha_s$ protein in GLP-1 receptor expressing membranes is measured by antibody capture scintillation proximity. Data are normalized to control values measured from saturating GLP-1 (7–36)NH_2 concentrations. Data are fit to the four parameter logistic equation. EC_{50} and E_{MAX} values (% of maximal GLP-1 (7–36)NH_2 effect): 0 μM BETP, 1.3 nM, 99%; 3 μM BETP, 1.3 nM, 95%; 10 μM 1.1 nM, 95 %. Data are the mean ± S.E.M. of a single experiment conducted in triplicate and are representative of three independent experiments.

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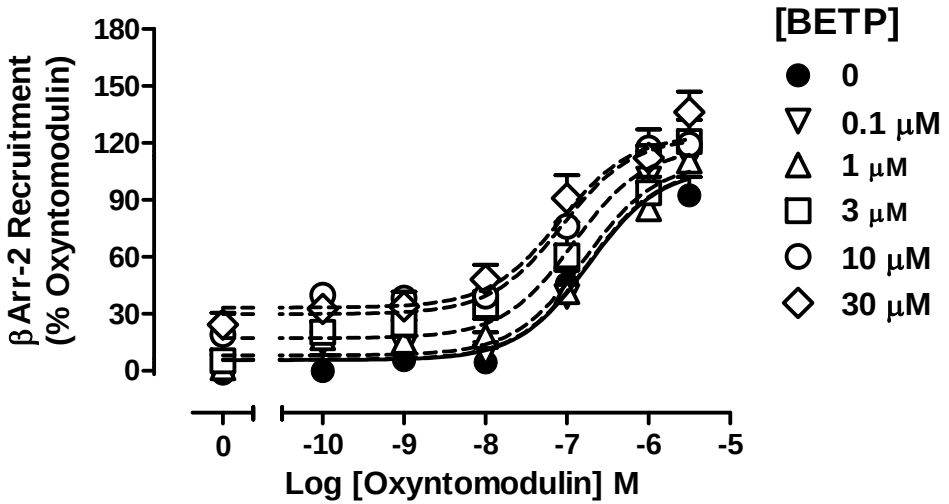

Supplementary Figure 4

BETP modulation of oxyntomodulin stimulated GLP-1 receptor mediated activation of β-arrestin2. The dose-dependent effects of BETP on oxyntomodulin mediated activation of β-arrestin2 recruitment are measured by bioluminescence resonance energy transfer. Data are fit to the operational model of allosteric agonism to quantify biased signaling; resultant calculated parameters are in Table 1.

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Assay	pIC_{50}		pEC_{50}		Emax (% GLP-1(7-36)NH_2)	
	- BETP	+ BETP	- BETP	+ BETP	- BETP	+ BETP
Binding	7.92 ± 0.10	8.82 ± 0.13	-	-	-	-
GTPγS	-	-	7.78 ± 0.03	8.40 ± 0.04	95 ± 1	100 ± 1
cAMP	-	-	8.48 ± 0.07	9.44 ± 0.11	98 ± 2	94 ± 3
pERK1/2	-	-	7.34 ± 0.17	6.93 ± 0.17	95 ± 8	83 ± 4
Ca^{2+}	-	-	6.34 ± 0.19	6.51 ± 0.22	105 ± 12	144 ± 9
β-Arr1	-	-	7.13 ± 0.16	7.48 ± 0.25	105 ± 7	147 ± 9
β-Arr2	-	-	6.92 ± 0.13	7.28 ± 0.18	101 ± 6	124 ± 7

Supplementary Table 1

Oxyntomodulin binding and signalling parameters in the absence and presence of 30 μM BETP. Data were analysed using a four parameter logistic equation as defined in materials and methods. pIC_{50} values represent the negative logarithm of the concentration of ligand that inhibits binding of half the total concentration of radiolabelled antagonist, ^{125}I-exendin(9-39). pEC_{50} values represent the negative alogarithm of the concentration of agonist that produces half the maximal response. Emax values represent the maximal response normalised to that elicited by 100 nM GLP-1(7-36)NH_2.