

# Small Molecule Allosteric Modulation of the Glucagon-Like Peptide-1 Receptor Enhances the Insulinotropic Effect of Oxyntomodulin<sup>[S]</sup>

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Received June 8, 2012; accepted August 28, 2012

## ABSTRACT

Identifying novel mechanisms to enhance glucagon-like peptide-1 (GLP-1) receptor signaling may enable nascent medicinal chemistry strategies with the aim of 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-benzoyloxyphenyl)-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 in-

creased oxyntomodulin binding affinity for the GLP-1 receptor and enhanced oxyntomodulin-mediated GLP-1 receptor signaling as measured by activation of the  $\alpha$  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 that GLP-1 receptor mobilization of cAMP is the critical insulinotropic signaling event. Because of the unique metabolic properties of oxyntomodulin, identifying molecules that enhance its activity should be pursued to assess the efficacy and safety of this novel mechanism.

## Introduction

The GLP-1 receptor mediates the predominant and best characterized physiological actions of oxyntomodulin (glucagon-37), a peptide generated by tissue-specific, post-transla-

tional processing of proglucagon (Mojsov 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 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

This work was supported in part by the National Health and Medical Research Council of Australia [Project Grant 1002180, Program Grant 519461].

F.S.W., D.W., and A.D.S. contributed equally to this work.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
<http://dx.doi.org/10.1124/mol.112.080432>.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

**ABBREVIATIONS:** GLP-1, glucagon-like peptide-1; CNS, central nervous system; GPCR, G protein-coupled receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; DPP-4, dipeptidyl peptidase-4; BETP, 4-(3-benzoyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; HEK, human embryonic kidney; CHO, Chinese hamster ovary; Rluc, *Renilla* luciferase; BRET, bioluminescence resonance energy transfer; GTP $\gamma$ S, guanosine 5'-O-(3-thio)triphosphate; IVGTT, intravenous glucose tolerance test; HHB, hypotonic homogenization buffer.

mice (Maida et al., 2008). In the CNS, the metabolic dependence of GLP-1 receptor signaling for oxyntomodulin efficacy is shown in feeding studies in which 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 et al., 2004). In addition to genetic ablation, studies using the peptide GLP-1 receptor antagonist, exendin-4(9–39), demonstrate that pharmacological blockade of the GLP-1 receptor attenuates oxyntomodulin-induced insulin secretion from isolated islets and INS-1 832/3 cells (Maida et al., 2008), and it blunts oxyntomodulin-mediated inhibition of food intake (Dakin et al., 2004).

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<sub>2</sub>/(7–37) are competitive ligands and full agonists of the GLP-1 receptor, although oxyntomodulin has lower binding affinity than GLP-1(7–36)-NH<sub>2</sub>/(7–37) (Fehmann et al., 1994; Baggio et al., 2004). Oxyntomodulin is also a biased agonist at the GLP-1 receptor relative to GLP-1(7–36)-NH<sub>2</sub>/(7–37), exhibiting less preference toward cAMP signaling relative to phosphorylation of ERK1/2, indicating that physiological responses to oxyntomodulin via the GLP-1 receptor could differ from those elicited by GLP-1(7–36)-NH<sub>2</sub>/(7–37) (Koole et al., 2010).

Although additional studies are needed to fully understand the physiological significance of endogenous oxyntomodulin acting on the GLP-1 receptor, both GLP-1(7–36)-NH<sub>2</sub>/(7–37) and oxyntomodulin are derived from the same precursor protein and cosecreted upon meal ingestion (Mojsov et al., 1986; Le Quellec et al., 1992). Of importance, differences in metabolic clearance of GLP-1(7–36)-NH<sub>2</sub>/(7–37) versus oxyntomodulin may enhance oxyntomodulin-mediated signaling at the GLP-1 receptor as a result of more rapid inactivation of GLP-1(7–36)-NH<sub>2</sub>/(7–37) by DPP-4 because it is a better DPP-4 substrate than oxyntomodulin (Zhu et al., 2003). The half-life of GLP-1(7–36)-NH<sub>2</sub>/(7–37) is 1 to 2 min (Siegel et al., 1999), whereas half-life estimates for oxyntomodulin range from 6 to 12 min (Baldissiera et al., 1988; Schjoldager et al., 1988; Kervran et al., 1990). Furthermore, infusion studies in humans confirm the metabolic actions of oxyntomodulin (Cohen et al., 2003), and new drug discovery approaches to develop long-acting analogs of oxyntomodulin are being pursued (Pocai et al., 2009; Santoprete et al., 2011). Whereas 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 low-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 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 et al., 2010; Wootten 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 that 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 that cAMP mobilization is the principal insulinotropic signal transduction pathway of the GLP-1 receptor.

## Materials and Methods

**Ligands.** BETP was synthesized at Eli Lilly and Company as reported previously (Sloop et al., 2010). GLP-1(7–36)-NH<sub>2</sub> and oxyntomodulin were either purchased (Bachem California, Torrance, CA) or generated on solid support using an automated peptide synthesizer and Fmoc protocols. After 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 before experiments were conducted.

**HEK293 and CHO Cellular Assays.** HEK293 cells transiently expressing the human GLP-1 receptor at 80,000 receptors/cell or the human glucagon receptor were used for measurement of cAMP accumulation. Cells were grown at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and 20 mM HEPES. Cells were transfected in suspension at  $6.25 \times 10^4$  cells/ml contained in supplemented medium lacking antibiotics with a 6:1 FuGENE6 (Promega, Madison, WI) transfection reagent/plasmid DNA ratio. Forty-eight hours after transfection, *i.e.*, 2 h before compound testing, cells were lifted, resuspended in 0.5% fetal bovine serum-supplemented (as above) Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), and kept at 37°C. Transfected cells were seeded at a density of 2500 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  $\mu$ M final concentration), were added to the cells. After a 20-min incubation, cells were assayed for cAMP using homogeneous time-resolved fluorescence (Cisbio, Bedford, MA) in 100- $\mu$ l reactions. Fluorescence was measured according to the manufacturer's instructions using an EnVision plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). Data were analyzed by the ratio method and calibrated to external standards and expressed as percent cAMP compared with the reference peptide agonists. For CHO cellular assays, Fip-In CHO cells expressing the human GLP-1 receptor at a density of 120,000 receptors/cell were used; intracellular Ca<sup>2+</sup> mobilization, ERK1/2 phosphorylation, and cAMP accumulation were measured as described previously (Wootten et al., 2012).

**$\beta$ -Arrestin Recruitment Assays.** The pE5-frt-V5 pDestination vector (Invitrogen) 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  $\beta$ -arrestin1- or  $\beta$ -arrestin2-Venus fusions. The native encephalomyocarditis virus internal ribosome entry site was chosen because this is reported to produce 7- to 10-fold more protein from the second cistron (the  $\beta$ -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<sub>1</sub> muscarinic acetylcholine receptor, demonstrating a comparable ligand-induced response to experiments in which 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, Fip-In CHO

cell lines stably expressing GLP-1 receptor-Rluc8 and either  $\beta$ -arrestin1- or  $\beta$ -arrestin2-Venus were generated using gateway technology. The fusion of Rluc8 to the C terminus of the GLP-1 receptor did not alter its pharmacology as assessed in cAMP accumulation, phosphorylated ERK1/2, and  $\text{Ca}^{2+}$  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 h. Cells were rinsed once with Hanks' balanced salt solution to remove traces of phenol red and incubated in fresh Hanks' balanced salt solution for a further 15 min. The Rluc substrate coelenterazine-h was added to reach a final concentration of 5  $\mu\text{M}$ . After a 5-min 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 to 505 and 515 to 555 nm windows using filters with the appropriate band pass. The BRET signal was calculated by subtracting the ratio of 515 to 555 nm emission over 465 to 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 min to determine the time at which  $\beta$ -arrestin1 and  $\beta$ -arrestin2 recruitment was maximal for each ligand in the absence or presence of BETP. Coaddition of ligands was performed for interaction assays, and BRET signals were collected at this peak time point.

**[ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  and [ $^{125}\text{I}$ ]-exendin-4(9–39) Binding Assays.** Membranes were prepared from HEK293 cells stably expressing the human GLP-1 receptor as described previously (Sloop et al., 2010). Cells were resuspended at 5 ml/g cell paste in HHB: 25 mM Tris-HCl, pH 7.5, 1 mM  $\text{MgCl}_2$ , and 1 $\times$  Complete inhibitors without EDTA (Roche, Indianapolis, IN). The cell suspension was initially disrupted in the presence of 25 U/ml DNase I with 20 to 25 strokes of a motorized, Dounce homogenizer and Teflon pestle and centrifuged at 1800g for 15 min 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 and centrifuged at 25,000g for 30 min. High-speed pellets were resuspended in 2 ml of HHB/g original cell paste and measured for protein content with bicinchoninic acid reagent (Pierce, Waltham, MA) and colorimetric detection. Receptor activation was measured via [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  binding to  $\text{G}\alpha_s$  using an antibody capture scintillation proximity assay (DeLapp et al., 1999). Reactions contained 50  $\mu\text{g}$  of membrane in 20 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM  $\text{MgCl}_2$ , 40  $\mu\text{g}/\text{ml}$  saponin, 0.1% bovine serum albumin, and  $^{35}\text{S}$ -labeled 500 pM guanosine 5'-( $\gamma$ -thio)triphosphate (PerkinElmer Life and Analytical Sciences). Peptide and allosteric modulator were diluted and cotreated to a final concentration of 1% dimethyl sulfoxide. Binding was induced for 30 min at ambient temperature before solubilization with 0.2% NP40 detergent, 2  $\mu\text{g}/\text{ml}$  rabbit anti- $\text{G}\alpha_{s/\text{olf}}$  polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and 1 mg of anti-rabbit polyvinyltoluene beads (PerkinElmer Life and Analytical Sciences). The detection mixtures were developed for 30 min, centrifuged at 80g for 10 min, and counted for 1 min/well using a MicroBeta TriLux instrument (PerkinElmer Life and Analytical Sciences). The GLP-1 receptor binding assay using [ $^{125}\text{I}$ ]-exendin-4(9–39) as the radioligand was performed as described previously (Wootten 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 Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). 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), group-housed at three per cage in polycarbonate cages with filter tops. Rats

were maintained on a 12-h light/dark cycle (lights on at 6:00 AM) at 21°C and received 2014 Teklad Global diet (Harlan) and deionized water ad libitum. Rats were fasted overnight and anesthetized with 60 mg/kg Nembutal (Lundbeck, Deerfield, IL) for the duration of the experiment. For glucose and compound administration, a catheter with a diameter of 0.84 mm (Braintree Scientific, Braintree, IL) was inserted into the jugular vein. For rapid blood collection, a larger catheter with 1.02-mm diameter (Braintree Scientific) was inserted into the carotid artery. Blood was collected for glucose and insulin levels at times 0, 2, 4, 6, 10, and 20 min 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).

**Data Analysis.** Pharmacology data were fit using Prism 5.0 (GraphPad Software Inc. (San Diego, CA)) 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 et al., 2007) to determine ligand cooperativity (eqs. 1 and 2). In this case, nondepletion of ligands was assumed (Avlani et al., 2008):

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

where

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

where  $Y$  represents radioligand binding,  $B_{\max}$  denotes maximal binding site density, and NS denotes the fraction of nonspecific binding.  $[A]$  and  $K_A$  denote the concentration of radioligand and the 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.  $\alpha$  and  $\beta$  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  $\alpha > 1$  are indicative of an allosteric-mediated increase in binding activity, whereas values of  $0 < \alpha < 1$  are indicative of an allosteric-mediated decrease in binding affinity. In cell signaling ligand interaction studies, data were fitted to the following two forms (eqs. 3 and 4) of an operational model of allosterism and agonism to derive functional estimates of modulator affinity and cooperativity (Leach et al., 2007; Aurelio et al., 2009):

$E =$

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

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

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,  $\alpha$  is the binding cooperativity factor,  $\beta$  is an empirical scaling factor describing the allosteric effect of the modulator on orthosteric agonist signaling efficacy, respectively, and  $\tau_A$



and  $\tau_B$  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 an allosteric ligand (BETP) and a partial agonist (intracellular  $\text{Ca}^{2+}$ ,  $\beta$ -arrestin1, and  $\beta$ -arrestin2), whereas eq. 4 was used when BETP interacted with a full agonist (GTP $\gamma$ S, cAMP, and phospho-ERK1/2), because eq. 2 is only valid in cases in which the orthosteric agonist has high efficacy ( $\tau \gg 1$ ) such that  $K_A$  is  $\gg [A]$ .

**Statistics.** All data are represented as means  $\pm$  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  $p < 0.05$ .

## Results

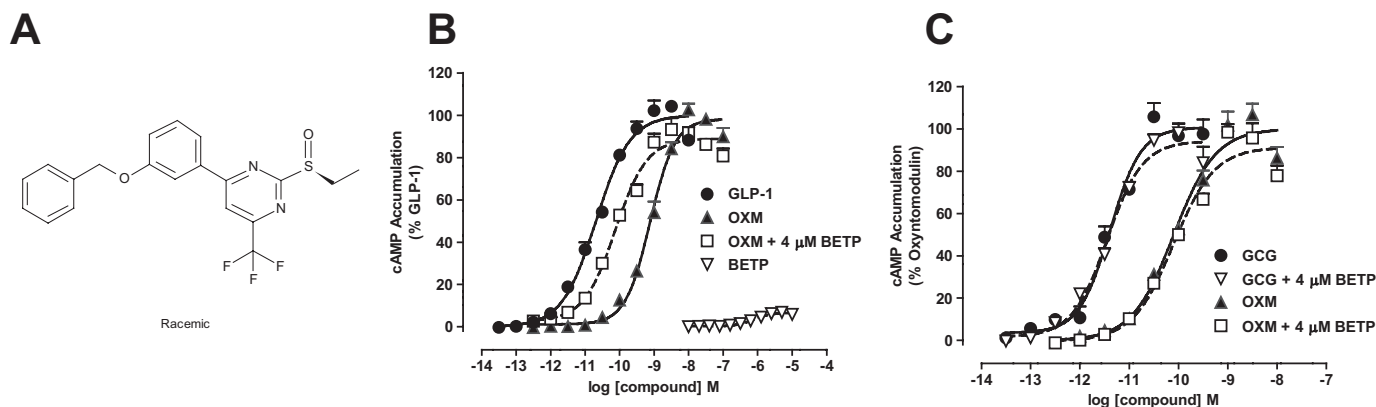
**BETP Potentiates Oxyntomodulin-Induced Signaling.** We previously demonstrated that BETP (Fig. 1A) is a GLP-1 receptor ligand with micromolar intrinsic partial agonism in pancreatic islets and in vivo (Sloop et al., 2010). Moreover, BETP is an effective positive allosteric modulator of the naturally occurring, inactive GLP-1 metabolite GLP-1(9–36)-NH<sub>2</sub> but shows little modulation of the active, circulating forms of GLP-1, GLP-1(7–36)-NH<sub>2</sub>/(7–37) (Wootten et al., 2012). Here, we hypothesized that BETP could be effective in potentiating endogenous GLP-1 receptor ligands with lower affinity than GLP-1(7–36)-NH<sub>2</sub>/(7–37), such as the comparatively low-affinity full agonist oxyntomodulin (Fehmann et al., 1994; Baggio 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 (Fig. 1B). In this system, GLP-1(7–36)-NH<sub>2</sub> is a highly potent full agonist ( $\text{EC}_{50} = 20$  pM), whereas oxyntomodulin is a full agonist with 40-fold reduced potency ( $\text{EC}_{50} = 800$  pM). In the presence of a submaximal concentration of BETP (4  $\mu\text{M}$ ), the potency of oxyntomodulin increases 10-fold ( $\text{EC}_{50} = 80$  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<sub>2</sub> (Wootten et al., 2012).

To assess whether the effect of BETP is specific and selec-

tive 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 glucagon, is a full agonist at the glucagon receptor with 20-fold lower potency ( $\text{EC}_{50} = 80$  pM) than the canonical ligand glucagon ( $\text{EC}_{50} = 4$  pM) (Fig. 1C). In the presence of 4  $\mu\text{M}$  BETP, the potencies and efficacies of both oxyntomodulin and glucagon at the glucagon receptor are unaltered, thus demonstrating that BETP is a GLP-1 receptor-selective positive allosteric modulator.

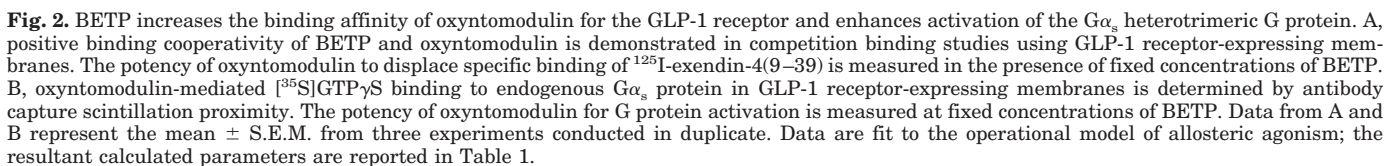
**BETP Increases GLP-1 Receptor Binding of Oxyntomodulin.** To determine the specific mechanism of BETP allosteric modulation of oxyntomodulin, we undertook radioligand binding studies to quantify the effect of BETP on oxyntomodulin affinity for the GLP-1 receptor. BETP dose-dependently increases the ability of oxyntomodulin to compete with <sup>125</sup>I-exendin-4(9–39) for binding to the GLP-1 receptor in a whole-cell binding assay (Fig. 2A). We used an operational model of allosteric agonism to quantify cooperativity between BETP and oxyntomodulin (Leach et al., 2007). BETP allosteric modulation of oxyntomodulin is affinity-driven with an  $\alpha$  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 GTP $\gamma$ S binding assay with membranes from GLP-1 receptor-expressing cells. In this system, the basal signal is approximately 2300 cpm, and the saturating GLP-1(7–36)-NH<sub>2</sub> signal is approximately 12,000 cpm (Supplemental Fig. 1). GLP-1(7–36)-NH<sub>2</sub> and oxyntomodulin are both full agonists with potencies close to their binding affinities (Druce and Bloom, 2006) (Supplemental Fig. 2). BETP dose-dependently increases the potency of oxyntomodulin-stimulated G protein activation (Fig. 2B), and, at saturation, this is within 2-fold of the observed potency of GLP-1(7–36)-NH<sub>2</sub> (Supplemental Table 1), indicating that BETP is capable of potentiating the functional effects of oxyntomodulin to be on par with GLP-1(7–36)-NH<sub>2</sub>. BETP alone is a low-potency partial agonist in the GTP $\gamma$ S binding



**Fig. 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  $\mu\text{M}$  BETP. Data are fit to the four-parameter logistic equation and calculated  $\text{EC}_{50}$  and  $E_{\text{max}}$  values [percentage of maximal GLP-1(7–36)-NH<sub>2</sub> effect] are as follows: GLP-1(7–36)-NH<sub>2</sub>, 21 pM, 100%; BETP, 740 nM, 7%; OXM, 770 pM, 98%; and OXM + 4  $\mu\text{M}$  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  $\mu\text{M}$  BETP. Data are fit to the four-parameter logistic equation.  $\text{EC}_{50}$  and  $E_{\text{max}}$  values (percentage of maximal OXM effect) are as follows: GCG, 4 pM, 101%; GCG + 4  $\mu\text{M}$  BETP, 4 pM, 94%; OXM, 80 pM, 100%; and OXM + 4  $\mu\text{M}$  BETP, 79 pM, 91%. All data in **B** and **C** are mean  $\pm$  S.E.M. of three to five independent experiments conducted in duplicate.

**BETP Enhances Oxyntomodulin-Induced Insulin Secretion.** To test whether BETP potentiation of oxyntomodulin could be observed in an intact physiological system, we

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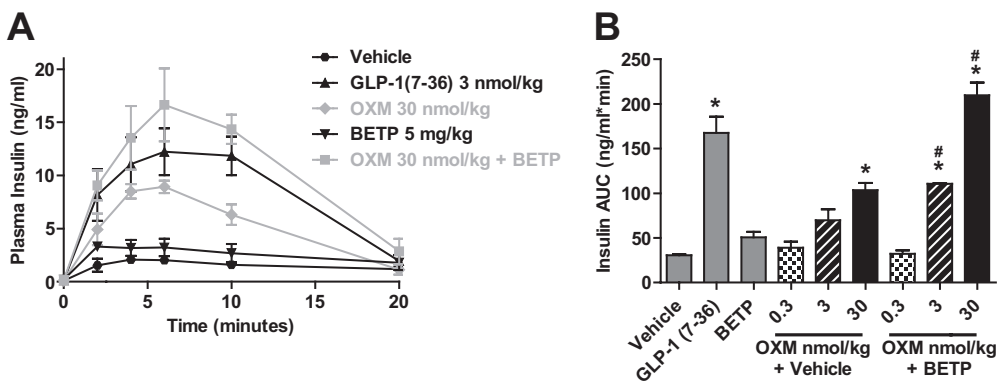
Allosteric model parameters describing the cooperativity for the interaction between BETP and oxyntomodulin

$\alpha$  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 eqs. 1 and 2.  $\alpha\beta$  is the cooperativity factor that defines the fold change in receptor signaling by BETP and is a composite factor describing the combined affinity ( $\alpha$ ) and efficacy ( $\beta$ ) modulation by the allosteric ligand. This is calculated using an operational model of agonism as defined in eqs. 3 and 4.  $\text{pIC}_{50}$ ,  $\text{pEC}_{50}$ , and  $E_{\max}$  values for these data sets are presented in Supplemental Table 1.

Assay Measurement	$\text{Log}\alpha$ ( $\alpha$ )	$\text{Log}\alpha\beta$ ( $\alpha\beta$ )	$\text{Log}\beta$ ( $\beta$ ) ( $\text{Log}\alpha\beta - \text{Log}\alpha$ )
Binding (affinity)	$1.16 \pm 0.10$ (14.6) <sup>*a</sup>	-	-
GTP $\gamma$ S		$1.11 \pm 0.05$ (12.9)*	$-0.05 \pm 0.11$ (0.89)
cAMP		$1.10 \pm 0.08$ (12.6)*	$-0.06 \pm 0.13$ (0.87)
Phospho-ERK1/2		$-0.44 \pm 0.19$ (0.36)	$-1.60 \pm 0.21$ (0.03)*
Ca <sup>2+</sup>		$0.23 \pm 0.11$ (1.70)	$-0.36 \pm 0.39$ (0.44)
$\beta$ -Arrestin1		$0.40 \pm 0.08$ (2.51)	$-0.76 \pm 0.13$ (0.17)
$\beta$ -Arrestin2		$0.54 \pm 0.19$ (3.47)	$-0.62 \pm 0.21$ (0.24)

\*Statistically significant at  $p < 0.05$ .

<sup>a</sup> The pK<sub>B</sub> value (the negative logarithm of the affinity) for BETP derived from application of the operational model of allosterism is 5.01 ± 0.09.



**Fig. 3.** Oxyntomodulin-stimulated insulin secretion is enhanced by BETP in vivo. **A**, time course of plasma insulin concentrations in fasted, anesthetized Wistar rats treated with either vehicle, GLP-1(7–36)-NH<sub>2</sub> (3 nmol/kg), oxyntomodulin (OXM) (30 nmol/kg), BETP (5 mg/kg), or OXM (30 nmol/kg) + BETP (5 mg/kg) immediately before intravenous administration of a glucose bolus (0.5 g/kg). **B**, integrated area under the curve (AUC) 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: \*,  $p < 0.05$ , versus vehicle; #,  $p < 0.05$ , OXM alone condition versus OXM + BETP treatment conditions. Data represent three experiments.

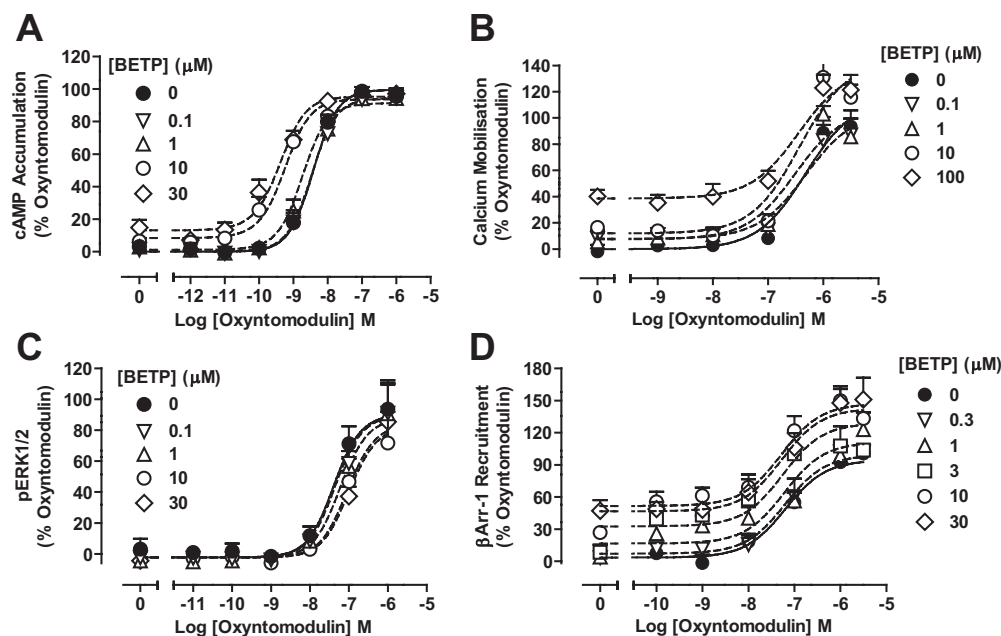
the GLP-1 receptor to promote insulin secretion (Maida et al., 2008). BETP at the concentration used in these studies causes only a minimal degree of insulin secretion above that of vehicle alone. However, at subsaturating concentrations of oxyntomodulin, the insulinotropic effect of oxyntomodulin is markedly enhanced by coadministration of BETP (Fig. 3). This effect is nonadditive [the difference in insulin area under the curve, mean  $\pm$  S.E.M. is as follows: BETP (5 mg/kg),  $20 \pm 6$  ng/ml  $\cdot$  min; oxyntomodulin (30 nmol/kg),  $73 \pm 8$  ng/ml  $\cdot$  min; and BETP (5 mg/kg) + oxyntomodulin (30 nmol/kg),  $179 \pm 14$ ] but 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<sub>2</sub> with varying degrees of positive and negative cooperativity for cAMP accumulation, ERK1/2 activation, and Ca<sup>2+</sup> mobilization (Wooten 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 (Fig. 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 (Fig. 4A; Table 1), whereas neutral cooperativity for Ca<sup>2+</sup> mobilization (Fig. 4B; Table 1) and efficacy-driven negative cooperativity for ERK1/2 activation ( $\beta = 0.03$ ) are observed (Fig. 4C; Table 1). The other major reported pathway of GLP-1 receptor signaling is via  $\beta$ -arrestin (Jorgensen et al., 2005). We measured oxyntomodulin-induced  $\beta$ -arrestin1 recruitment using BRET (Fig. 4D). Oxyntomodulin is a potent agonist of  $\beta$ -arrestin1 recruitment with potency and efficacy equivalent to those of GLP-1(7–36)-NH<sub>2</sub> (Supplemental Table 1); likewise, BETP shows intrinsic partial agonism for  $\beta$ -arrestin1 recruitment ( $E_{\max} = 45\%$  of

GLP-1(7–36)-NH<sub>2</sub>). Interaction experiments indicate that BETP and oxyntomodulin are neutrally cooperative for  $\beta$ -arrestin1 recruitment but additive in nature (Table 1). An interesting finding is that BETP enhances the efficacy of GLP-1 receptor-mediated  $\beta$ -arrestin1 signaling, suggesting that oxyntomodulin and GLP-1(7–36)-NH<sub>2</sub> are only partial agonists in this system (Supplemental Table 1). Equivalent results are observed using the GLP-1 receptor and  $\beta$ -arrestin2 (Supplemental Fig. 4). These data therefore indicate that BETP allosteric modulation of oxyntomodulin in heterologous systems is strongly biased toward cAMP accumulation and neutral toward  $\beta$ -arrestin recruitment, and, although not statistically significant, there is a trend toward negative bias for ERK1/2 activation (Table 1).

## Discussion

Increasing the concentration of endogenous, active GLP-1(7–36)-NH<sub>2</sub>/(7–37) by orally administered DPP-4 inhibitors is a proven and effective therapeutic approach for improving glucose control in patients with type 2 diabetes mellitus (Nauck et al., 2007). However, treatment with injectable GLP-1 receptor agonist peptides provides additional therapeutic benefits over treatment with molecules of the DPP-4 inhibitor class because the GLP-1 analogs elicit larger percentage hemoglobin A<sub>1c</sub> reductions and often lead to significant weight loss (DPP-4 inhibitors are weight neutral) (Buse et al., 2004; DeFronzo et al., 2005; Kendall et al., 2005). The more profound metabolic efficacy shown by peptide-based molecules occurs as a result of delayed gastric emptying, reduced postprandial hyperglucagonemia, and improved energy metabolism (DeFronzo et al., 2008). These effects are GLP-1 receptor-dependent (Hansotia et al., 2007; Lamont 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<sub>2</sub>/(7–37) (DeFronzo et al., 2008). In contrast, treatment with the DPP-4 inhibitor, sitagliptin,



**Fig. 4.** BETP induces cAMP biased signaling at the oxyntomodulin-bound GLP-1 receptor. The dose-dependent effects of BETP on oxyntomodulin-mediated activation of (A) cAMP accumulation, (B) intracellular Ca<sup>2+</sup> mobilization, (C) ERK1/2 phosphorylation, and (D)  $\beta$ -arrestin recruitment are quantified in Flp-In CHO cells expressing the GLP-1 receptor. Data are fit to the operational model of allosteric agonism to quantify biased signaling; the resultant calculated parameters are reported in Table 1.



raises the concentration of circulating GLP-1(7–36)-NH<sub>2</sub>/(7–37) by only 2-fold (Herman et al., 2005; DeFronzo et al., 2008). These data indicate that higher exposure of GLP-1 receptor agonist improves treatment efficacy and thus suggests that there is additional therapeutic capacity by which orally available small molecules can enhance GLP-1 receptor activation beyond that achieved by DPP-4 inhibition.

Several groups have recently reported efforts to identify and explore development of nonpeptide, orally available GLP-1 receptor agonists or positive allosteric modulators (for a review, see Willard et al., 2012). Whereas discovery of surrogate agonists that use a receptor binding and activation mechanism similar to GLP-1 is probably difficult, we have reported that small molecules acting allosterically may be a more feasible approach (Koole et al., 2010; Wootten 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 postprandial kinetic profile of oxyntomodulin secretion, its half-life, and its GLP-1 receptor-binding properties. Similar to GLP-1(7–36)-NH<sub>2</sub>/(7–37), oxyntomodulin is released from endocrine L cells in the gut after meal ingestion (Le Quellec et al., 1992), an important metabolic period during which glucose-stimulated insulin secretion is needed to effectively reduce postprandial 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<sub>2</sub>/(7–37) (Baldissera et al., 1988; Schjoldager et al., 1988; Kervran et al., 1990). In addition, oxyntomodulin is a full GLP-1 receptor agonist, although its binding affinity is lower for the GLP-1 receptor than for GLP-1(7–36)-NH<sub>2</sub>/(7–37) (Fehmann et al., 1994; Baggio et al., 2004).

Of importance, this report shows proof of concept that a small molecule approach to potentiate oxyntomodulin activity on the GLP-1 receptor can be exploited 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<sub>α<sub>s</sub></sub> 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 pursuing 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 because we only observe GLP-1 receptor-dependent action by this receptor when tested against a number of class B GPCRs (Sloop 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 antihyperglycemic 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<sup>2+</sup> 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 et al., 2010). The finding that two structurally distinct molecules show similar pharmacological effects on cAMP signaling is important for nascent efforts aimed to discover novel potentiator chemotypes. Whereas the *in vivo* effect of BETP on acute insulin secretion supports the hypothesis that modulating oxyntomodulin action improves glucose metabolism, unfortunately, the physiochemical liabilities of BETP and compound 2 (both are unstable in the presence of nucleophiles) limit longer term studies (Teng et al., 2007; Willard et al., 2012). For example, additional experiments are needed to explore the overall therapeutic consequence of preferentially enhancing cAMP signaling versus other pathways. Likewise, 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 et al., 2009; Santoprete 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 because data show that mice orally dosed with the molecule display enhanced insulin secretion in an IVGTT (Kim et al., 2010; Moon et al., 2011). Thus, exploitation of biased GLP-1 receptor agonism using allosteric modulators to potentiate oxyntomodulin represents a novel theoretical approach for developing antidiabetic agents. The use of focused medicinal chemistry and relevant pharmacological approaches appears to represent the clearest path to testing this hypothesis.

Although BETP and compound 2 are not likely to advance into clinical testing, pharmacological characterization of these molecules demonstrates several attractive features that may have an impact on 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<sub>2</sub>/(7–37) for receptor binding (Knudsen et al., 2007; Sloop et al., 2010). Furthermore, we recently showed that these molecules also activate the GLP-1 receptor by potentiating the DPP-4 cleaved, inactive metabolite GLP-1(9–36)-NH<sub>2</sub>, but not the parent agonist GLP-1(7–36)-NH<sub>2</sub> (Wootten 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 physiological 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<sub>2</sub>, and, therefore, the two agonists may induce different physiological profiles. Furthermore, 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 en-

hance 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 milieu of GLP-1 receptor ligands released by incretin secretagogues with GLP-1 receptor potentiators may enhance the emerging secretagogue approach.

## Acknowledgments

We thank Wendy Gough and Brad Wainscott for helpful suggestions with GTP $\gamma$ S assay development.

## Authorship Contributions

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

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

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

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

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

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