Allosteric Modulation of Endogenous Metabolites as an Avenue for Drug Discovery⑤

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ABSTRACT

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors and a key drug target class. Recently, allosteric drugs that can cobind with and modulate the activity of the endogenous ligand(s) for the receptor have become a major focus of the pharmaceutical and biotechnology industry for the development of novel GPCR therapeutic agents. This class of drugs has distinct properties compared with drugs targeting the endogenous (orthosteric) ligand-binding site that include the ability to sculpt cellular signaling and to respond differently in the presence of discrete orthosteric ligands, a behavior termed "probe dependence." Here, using cell signaling assays combined with ex vivo and in vivo studies of insulin secretion, we demonstrate that allosteric ligands can cause marked potentiation of previously "inert" metabolic products of neurotransmitters and peptide hormones, a novel consequence of the phenomenon of probe dependence. Indeed, at the muscarinic M_2 receptor and glucagon-like peptide 1 (GLP-1) receptor, allosteric potentiation of the metabolites, choline and GLP-1(9–36)NH$_2$, respectively, was ~100-fold and up to 200-fold greater than that seen with the physiological signaling molecules acetylcholine and GLP-1(7–36)NH$_2$. Modulation of GLP-1(9–36)NH$_2$ was also demonstrated in ex vivo and in vivo assays of insulin secretion. This work opens up new avenues for allosteric drug discovery by directly targeting modulation of metabolites, but it also identifies a behavior that could contribute to unexpected clinical outcomes if interaction of allosteric drugs with metabolites is not part of their preclinical assessment.

Introduction

G protein-coupled receptors (GPCRs) are the largest superfamily of cell surface proteins and play crucial roles in virtually every physiological process. Their widespread abundance and ability to couple to a variety of signaling and effector systems make them extremely attractive targets for drug development (Christopoulos, 2002). GPCR agonist drug discovery efforts have traditionally focused on either increasing the endogenous orthosteric agonist concentration by inhibiting its breakdown or targeting the orthosteric binding site of the receptor with surrogate agonists. However, in recent years there has been a significant increase in the identification of small molecules that target topographically distinct allosteric sites on GPCRs (May et al., 2007b). Binding of allosteric ligands can elicit a conformational change in the receptor while still allowing the orthosteric ligand to bind, thus modulating the pharmacological properties (affinity and/or efficacy) of the orthosteric ligand, in addition to

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; LUF6000, N-(3,4-dichlorophenyl)-2-cyclohexyl-1H-imidazo[4,5-c]quinolin-4-amine; A_3-AR, adenosine receptor subtype 3; M_2 mAChR, muscarinic acetylcholine receptor, subtype 2; A_1-AR, adenosine receptor subtype 1; ERK1/2, extracellular signal-regulated kinase 1 and 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LY2033298, 3-amino-5-chloro-N-cyclopropyl-6-methoxy-4-methyl-thieno[2,3-b]pyridine-2-carboxamide; Compound 2, 6,7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; BETP, (4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; VCP171, (2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone; CHO, Chinese hamster ovary; ACh, acetylcholine; Ch, choline; PD81723, (2-amino-4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone; GTPγS, guanosine 5′-O-(3-thio)triphosphate; IVGTT, intravenous glucose tolerance test; pERK1/2, extracellular signal-related kinase 1 and 2 phosphorylation; DM, diabetes mellitus.

potentially activating the receptor in the absence of orthosteric ligand. Allosteric drugs have substantial potential as therapeutic agents, because they can provide novel receptor selectivity, in addition to offering the possibility of "fine tuning" existing physiological responses while maintaining the spatial and temporal characteristics of innate endogenous signaling (Christopoulos and Kenakin, 2002).

One characteristic of allostery is the phenomenon of "probe dependence," whereby the extent and direction of an allosteric interaction varies with the nature of the orthosteric ligand occupying the receptor (Kenakin, 2005). Furthermore, biased signaling leading to pathway-selective allosteric modulation can also result (Leach et al., 2007). These concepts are particularly relevant to receptor systems that have multiple endogenous ligands, such as the glucagon-like peptide-1 receptor (GLP-1R) system (Baggio and Drucker, 2007), because probe dependence can lead to different endogenous agonists of the same GPCR being allosterically modulated in strikingly different ways (Koole et al., 2010). However, a hitherto-unappreciated extension of this phenomenon is the possibility that endogenous metabolites of GPCR agonists, which may normally be minimally active in their own right, can also be influenced by allosteric modulators. Indeed, a recent study reported that the allosteric compound N-(3,4-dichlorophenyl)-2-cyclohexyl-1H-imidazo[4,5-c]quinolin-4-amine (LUF6000) can enhance signaling by inosine (the metabolite of adenosine) at the adenosine A_3 receptor (A_3-AR) (Gao et al., 2011). Although drug discovery programs focusing on developing small molecule allosteric drugs invariably screen for compounds that modulate responses mediated by the predominant orthosteric receptor agonist, it is currently not routine to incorporate similar studies on endogenous metabolites. However augmentation of metabolite signaling could offer a new therapeutic avenue for development of novel drugs, especially in systems in which the endogenous ligand is rapidly degraded to its (ostensibly) inactive metabolite (Fig. 1).

In this study, we investigated the potential to allosterically modulate the activity of the predominant, inactive metabolite of the physiological ligand at three different GPCRs for which small molecule allosteric modulators have been described: the GLP-1R (Knudsen et al., 2007; Koole et al., 2010; Sloop et al., 2010), the M_2 muscarinic acetylcholine receptor (M_2 mAChR) (Valant et al., 2012), and the adenosine A_1 receptor (A_1-AR) (Bruns and Fergus, 1990) (Supplemental Fig. 1). In each instance, we find a significant degree of allosteric potentiation of the endogenous metabolite by the allosteric modulator. Moreover, for the GLP-1R, we also provide evidence of the allosteric modulator engendering biased signaling in terms of enhancing cAMP signaling mediated by the metabolite, while having little effect on extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation or intracellular Ca^{2+} mobilization. Ex vivo studies using static cultures of rat pancreatic islets, as well as in vivo experiments also revealed that allosteric modulation of the GLP-1 metabolite resulted in glucose-dependent insulin secretion. To our knowledge, this is the first study to explore the potential to allosterically modulate endogenous metabolites of multiple GPCR ligands at their respective receptors. The outcomes could have significant implications in development and screening of novel therapeutic agents in drug discovery programs.
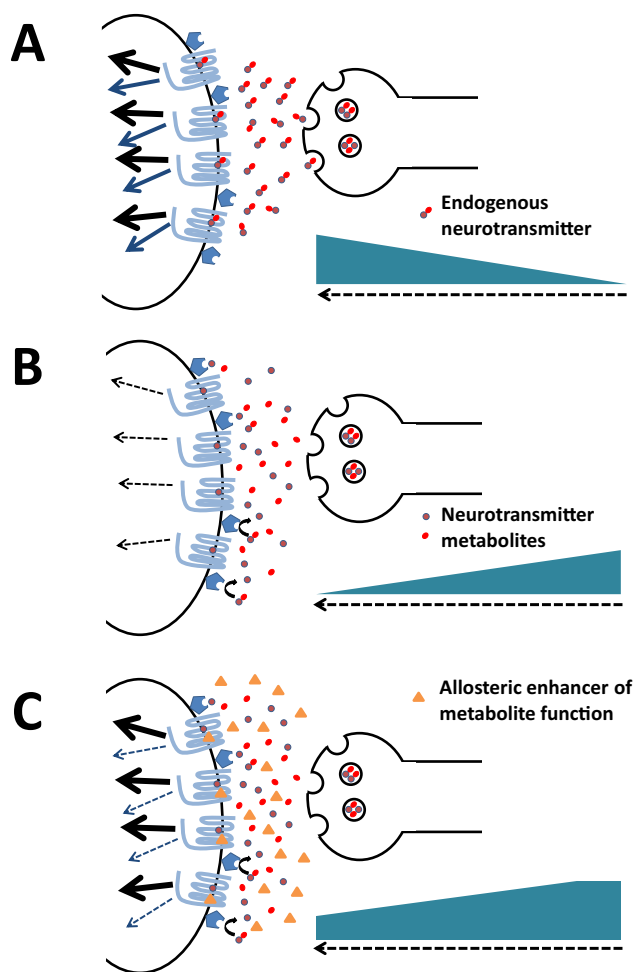

Fig. 1. Allosteric enhancement of metabolite activity as a novel mechanism of drug action. A, schematic illustration of neurotransmitter release and activation of postsynaptic receptors to elicit physiological signaling. B, the neurotransmitter is rapidly degraded by metabolizing enzymes, leading to decay of neurotransmitter signaling. C, an allosteric enhancer of metabolite activity (orange triangles) cobinds with the metabolite engendering and/or enhancing signaling to extend the activation of the receptor. A similar process can be envisaged for rapidly metabolized hormones or paracrine regulators.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), hygromycin B, and Fluo-4 acetoxymethyl ester were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Melbourne, VIC, Australia). AlphaScreen reagents, ^{125}I-labeled Bolton-Hunter reagent, and 384-well ProxiPlates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).SureFire ERK1/2 reagents were generously provided by TGR BioSciences (Adelaide, SA, Australia). 6,7-Dichloro-2-methylsulfonyl-3-*tert*-butylaminoquinoxaline (compound 2) was generated according to a method published previously (Teng et al., 2007) to a purity of >95%, and compound integrity was confirmed by NMR. (4-(3-Benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine (BETP) and 3-amino-5-chloro-N-cyclopropyl-6-methoxy-4-methyl-thieno[2,3-b]pyridine-2-carboxamide (LY2033298) were provided by Eli Lilly and GLP-1 peptides were purchased from American Peptide (Sunnyvale, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) or BDH Merck (Melbourne, VIC, Australia) and were of an analytical grade.

Transfections and Cell Culture

GLP-1R, M_2 mAChR, and A_1-AR were isogenically integrated into FlpIn-Chinese hamster ovary (FlpInCHO) cells (Invitrogen), and selection of receptor-expressing cells was accomplished by treatment with 600 μg/ml hygromycin B as described previously. Transfected and parental FlpInCHO cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37°C in 5% CO_2. For all whole-cell assays, cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates and incubated overnight at 37°C in 5% CO_2 before assaying.

Radioligand Binding Assay

GLP-1R Experiments. Growth medium was replaced with binding buffer [DMEM containing 25 mM HEPES and 0.1% (w/v) bovine serum albumin] containing 0.5 nM ^{125}I-exendin(9–39) and increasing concentrations of unlabeled peptide in the presence and absence of increasing concentrations of allosteric ligand. Cells were incubated overnight at 4°C, followed by three washes in ice-cold phosphate-buffered saline to remove unbound radioligand. Then 0.1 M NaOH was added, and radioactivity was determined by gamma counting. For GLP-1R experiments, nonspecific binding was defined by 1 μM exendin(9–39).

M_2 mAChR Experiments. M_2 mAChR FlpInCHO membrane homogenates (5–20 μg) were incubated in a 500-μl total volume of assay buffer containing [^{3}H]N-methylscopolamine (0.5 nM) with a range of concentrations of choline in the absence and presence of LY2033298 (1 and 10 μM) at 30°C for 90 min. All assays were performed in the presence of guanosine-5′-($\beta\gamma$-imino)triphosphate. For all experiments, nonspecific binding was defined by 10 μM atropine, and the effects of vehicle were also determined. Incubation was terminated by rapid filtration through Whatman GF/B filters using a cell harvester (Brandel Inc., Gaithersburg, MD). Filters were washed three times with 3-ml aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 ml of scintillation mixture (Ultima-Gold; PerkinElmer Life and Analytical Sciences). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined in disintegrations per minute using scintillation counting.

cAMP Assays

cAMP accumulation assays were performed using the AlphaScreen SureFire kit as described previously (Koole et al., 2010). Cells were stimulated with peptide ligand and/or allosteric ligand and incubated for 30 min at 37°C in 5% CO_2. cAMP accumulation was measured after 30 min of cell stimulation. All values were converted to concentration of cAMP, and data were subsequently normalized to the maximum response elicited by GLP-1(7–36)NH_2.

ERK1/2 Phosphorylation Assay

Receptor-mediated ERK1/2 phosphorylation was determined by using the AlphaScreen ERK1/2 SureFire protocol as described previously (May et al., 2007a). Initial ERK1/2 phosphorylation time course experiments were performed over 1 h to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by agonists. For GLP-1R, all responses peaked at 7 min; for M_2 mAChR, ACh, and Ch, responses peaked at 5 min, and for LY2033298, responses peaked at 8 min. For A_1-AR, adenosine and inosine peaked at 5 min, and (2-amino-4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone (PD81723) and (2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone (VCP171) peaked at 7 min. Subsequent concentration-response curves were constructed at the peak time point for each receptor/ligand combination.

Intracellular Ca^{2+} Mobilization Assay

Intracellular Ca^{2+} mobilization was determined as described previously (Werry et al., 2005). Fluorescence was determined immediately after drug addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings were taken every 1.36 s for 120 s. Concentration-response curves were constructed from the peak response, calculated using five-point smoothing, followed by correction against basal fluorescence.

[^{35}S]GTPγS Binding Assay

[^{35}S]GTPγS binding was determined as described previously (Valant et al., 2012). M_2 mAChR FlpInCHO cell membranes (5–25 μg) were equilibrated in a 500-μl total volume of assay buffer containing 10 μM guanosine 5′-diphosphate and a range of concentrations of ligands (ACh or Ch) in the absence or presence of LY2033298 (0.1–10 μM) at 30°C for 60 min. After this time, 50 μl of [^{35}S]GTPγS (1 nM) was added, and incubation continued for 30 min at 30°C. Incubation was terminated by rapid filtration through Whatman GF/B filters using a cell harvester (Brandell, Gaithersburg, MD). Filters were washed three times with 3-ml aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 ml of scintillation mixture (Ultima-Gold). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined in disintegrations per minute using scintillation counting.

Ex Vivo Pancreatic Islet Assays and In Vivo IVGTT Studies

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).

Ex Vivo Pancreatic Islet Assays

The procedures for isolating islets and performing the insulin secretion assays were described previously (Sloop et al., 2010). Islets were isolated from pancreases of male Sprague-Dawley rats using Hanks' balanced salt solution buffer (Sigma-Aldrich) containing 2% bovine serum albumin (Applichem, Boca Raton, FL) and 1 mg/ml collagenase (Sigma-Aldrich). Islets were purified using Histopaque (Histopaque-1077-Histopaque-11991 mixture; Sigma-Aldrich) gradients and cultured overnight in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). For the insulin secretion assays, islets were cultured at 37°C for 90 min in Earle's balanced salt solution (Invitrogen) containing the indicated concentrations of glucose and treatment conditions. Insulin that was released into the medium was measured using homogeneous time-resolved fluorescence technology (Cisbio Bioassays, Bedford, MA).

In Vivo IVGTT Studies

Male Wistar rats were purchased from Harlan (Indianapolis, IN) and maintained on a 12-h light/dark cycle at 21°C. For the studies, rats were fasted overnight and anesthetized the next morning with 60 mg/kg Nembutal (Lundbeck, Deerfield, IL). Catheters were then surgically inserted into the jugular vein and carotid artery for compound and/or peptide infusions and blood collection, respectively. For animal treatment, BETP was solubilized in a dosing solution containing 10% ethanol-Solutol, 20% polyethylene glycol 400, and 70% phosphate-buffered saline, pH 7.4, and infused intravenously alone or in combination with GLP-1(9–36)-NH_2 (Bachem California, Torrance, CA) formulated in saline containing 0.1% albumin. Blood was collected to determine glucose, insulin, and total GLP-1 levels after administration of an intravenous glucose bolus of 0.5 g/kg. Plasma levels of glucose were measured using a Hitachi 912 clinical chemistry analyzer (Roche, Indianapolis, IN), and insulin and total GLP-1 levels were determined using electrochemiluminescence assays for each (Meso Scale, Gaithersburg, MD).

Data Analysis

All data obtained were analyzed in GraphPad Prism 5.0.2 (GraphPad Software Inc., San Diego, CA). Radioligand inhibition binding data were fitted to a one-site inhibition mass action curve. Where

possible, in whole-cell ligand interaction studies, data were fitted to the following two forms of an operational model of allosterism and agonism (Leach et al., 2007; Aurelio et al., 2009) to derive functional estimates of modulator affinity and cooperativity.

$$E =$$

$$\frac{E_\text{m}(\tau_A[A](K_\text{B} + \alpha\beta[B]) + \tau_B[B]K_\text{A})^n}{([A]K_\text{B} + K_\text{A}K_\text{B} + [B]K_\text{A} + \alpha[A][B])^n + (\tau_A[A](K_\text{B} + \alpha\beta[B]) + \tau_B[B]K_\text{A})^n}$$

(1)

$$E = \frac{E_\text{m}(\tau_A[A](K_\text{B} + \alpha\beta[B]) + \tau_B[B]\text{EC}_{50})^n}{\text{EC}_{50}{}^n(K_\text{B} + [B])^n + (\tau_A[A](K_\text{B} + \alpha\beta[B]) + \tau_B[B]\text{EC}_{50})^n}$$ (2)

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 1 was used in interaction studies performed between allosteric ligand and a partial agonist, whereas eq. 2 was used when the modulator was interacted with full agonists, depending on the pathway investigated. This is so because eq. 2 is only valid in cases where the orthosteric agonist has high efficacy ($\tau \gg 1$) such that K_A is $\gg [A]$. For all other data, concentration-response curves were fitted with a three-parameter logistic equation.

Results

Allosteric Modulation of GPCR Agonist Metabolites Is Potentially a Widespread Phenomenon. To validate our hypothesis that metabolites of endogenous ligands can be

allosterically modulated at the GPCR of the parental ligand, we performed an initial screen using a representative allosteric ligand for three different model systems: the M_2 mAChR, the A_1-AR, and the GLP-1R. In a recent study, we characterized LY2033298 as an allosteric modulator of the M_2 mAChR (Valant et al., 2012). PD81723 is a well accepted allosteric modulator of the A_1-AR (Bruns and Fergus, 1990), and we have also recently identified a series of low-molecular-weight pyrimidine-based compounds that activate the GLP-1R allosterically, the most potent representative being BETP (designated compound B in Sloop et al., 2010). These three ligands (Supplemental Fig. 1D) were selected as representative modulators for each receptor, respectively. Both the M_2 mAChR and the A_1-AR are predominantly coupled to $G\alpha_i$ proteins, whereas the GLP-1R is primarily coupled to $G\alpha_s$. Therefore, in the initial screen ERK1/2 phosphorylation was assessed for both the M_2 mAChR and the A_1-AR, whereas cAMP accumulation assays were performed for the GLP-1R. All data were analyzed using an operational model of allosterism to derive global cooperativity estimates [$\alpha\beta$, a composite cooperativity factor quantifying allosteric modulation of the orthosteric ligand affinity (α) and efficacy (β)] (Table 1).

The cognate agonist for the M_2 mAChR, ACh, is rapidly converted to its inactive metabolites, Ch, and acetate, in the synaptic cleft by acetylcholinesterase (Birks and Macintosh, 1957) (Supplemental Fig. 1A). In this study, Ch exhibited greater than 1000-fold lower potency in ERK1/2 phosphorylation compared with the parent agonist ACh (Fig. 2, A and B). However, LY2033298 strongly potentiated the ERK1/2 response of Ch to a greater extent (112-fold) than that of ACh itself (Fig. 2, A and B; Table 1). In addition, assessment using a more proximal assay of M_2 mAChR activation (GTPγS binding) revealed LY2033298 potentiated the response to both ACh and Ch, but this effect was much greater for the metabolite (Supplemental Fig. 2;

TABLE 1
Allosteric parameters determining the cooperativity for the interaction between the allosteric modulators and agonist/metabolite at the three different GPCRs, using various signal outputs

Data were analyzed with an operational model of allosterism as defined under *Materials and Methods*. Log$\alpha\beta$ values represent the composite cooperativity between the allosteric modulator and the orthosteric ligand. Antilogarithms are shown in parentheses. pK_B values (the negative logarithm of the affinity) for the allosteric ligands derived from application of the operational model of allosterism were 5.01 ± 0.23 for BETP, 5.14 ± 0.16 for Compound 2, and 4.58 ± 0.32 for PD81723. For LY2033298, the pK_B was fixed to the equilibrium dissociation constant (4.74) previously determined in radioligand binding assays (Valant et al., 2012). $\alpha\beta$ is the cooperativity factor that defines the fold change in receptor signaling by the allosteric modulator.

| Allosteric Ligand and Signaling Pathway | Log $\alpha\beta$ ($\alpha\beta$) | | | | | |
| | Orthosteric Ligand | | | Orthosteric Metabolite | | |
	GLP-1(7–36)NH₂	ACh	Adenosine	GLP-1(9–36)NH₂	Ch	Inosine
GLP-1R						
BETP						
cAMP	0.18 ± 0.15 (1.5)			N.D.[a]		
pERK1/2	−0.97 ± 0.39 (0.1)			−0.01 ± 0.11 (1.0)		
Ca²⁺	N.D.[a]			N.D.		
Compound 2						
cAMP	0.36 ± 0.14 (2.3)			2.63 ± 0.43 (426)		
pERK1/2	−0.27 ± 0.26 (0.53)			0.25 ± 0.31 (1.8)		
Ca²⁺	N.D.			N.D.		
M₂ mAChR						
LY2033298						
pERK1/2		0.31 ± 0.07 (2.0)			2.35 ± 0.16 (224)	
GTPγS		1.20 ± 0.08 (16)			1.85 ± 0.10 (71)	
A₁-AR						
PD81723						
pERK1/2			1.31 ± 0.12 (20)			1.08 ± 0.12 (12)

N.D., data were not able to be experimentally defined.
[a] Cooperativity factors could not be defined, but positive allosteric modulation was observed.
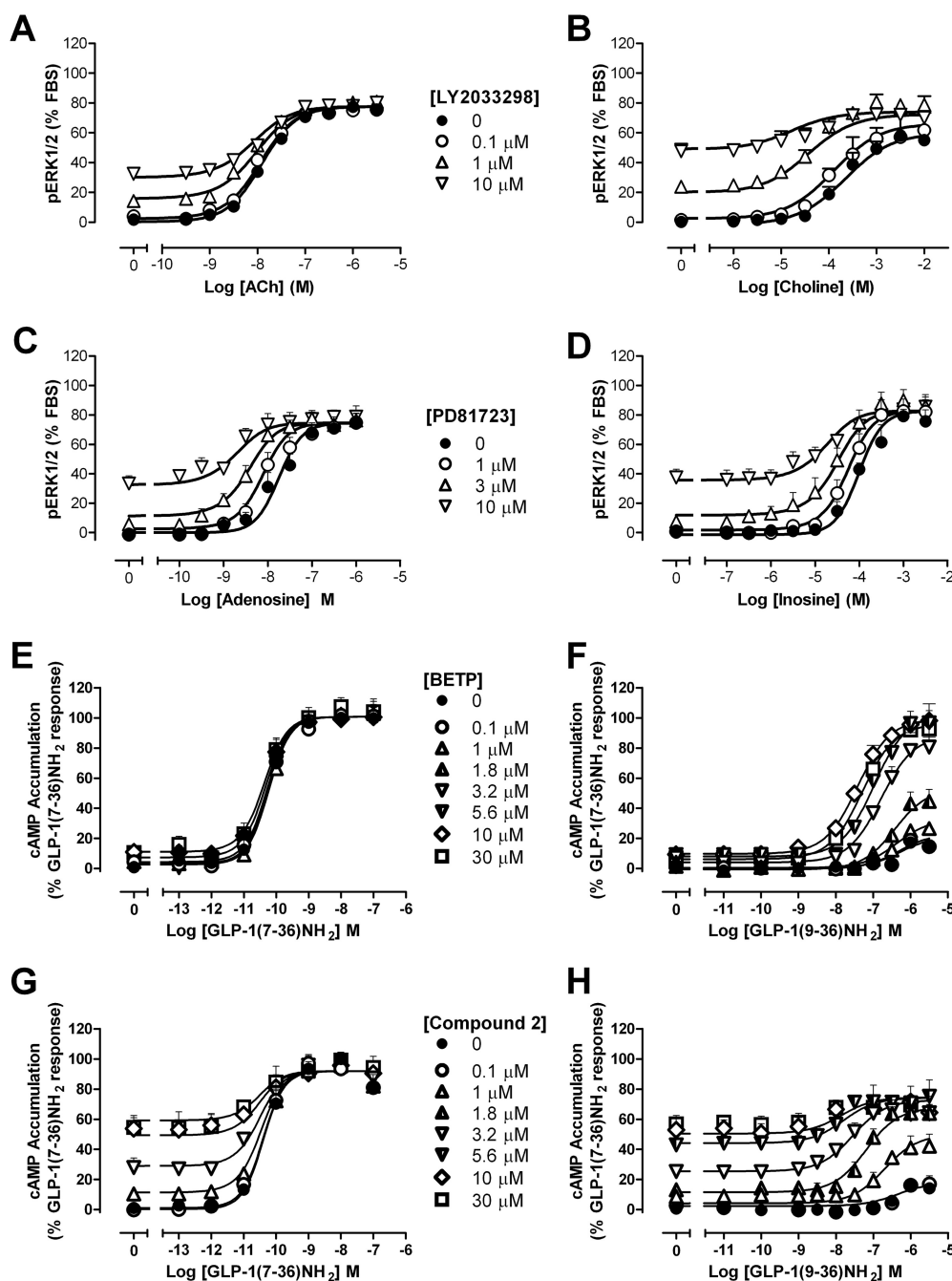
Fig. 2. Small-molecule ligands of three different GPCRs display a high degree of positive allosteric modulation of the metabolite of the cognate ligand in intact cells. Interaction studies were performed in ERK1/2 phosphorylation assays between LY2033298 and ACh (A) or Ch (B) in FlpInCHO cells stably expressing the human M_2 mAChR. Interaction studies between PD81723 and adenosine (C) or inosine (D) were performed in ERK phosphorylation assays in FlpInCHO cells expressing the A_1-AR. cAMP accumulation interaction studies were performed between BETP and GLP-1(7–36)NH_2 (E) or GLP-1(9–36)NH_2 (F) or between compound 2 and GLP-1(7–36)NH_2 (G) or GLP-1(9–36)NH_2 (H) in FlpInCHO cells stably expressing the human GLP-1R. All values are means ± S.E.M. of three to six independent experiments performed in duplicate.

Table 1). Competition binding assays revealed weak positive modulation of Ch affinity (13-fold) (Supplemental Fig. 3), indicating that potentiation of Ch in functional assays is principally via efficacy modulation. In our earlier study, we showed that LY2033298 also displayed positive cooperativity with ACh in binding affinity (16-fold) indicating that there is no efficacy modulation by LY2033298 with the parent agonist (Valant et al., 2012). Similarly to ACh, adenosine is also rapidly metabolized (by adenosine deaminase) to inosine (Plagemann et al., 1985) (Supplemental Fig. 1B); inosine displayed greater than 1000-fold lower potency at the A_1-AR in ERK1/2 phosphorylation compared with its parent ligand, adenosine. In addition, this response was significantly potentiated by the allosteric modulator, PD81723 (Fig. 2, C and D); although in this

instance the degree of potentiation was no greater than that observed with adenosine (Table 1). At the GLP-1R, BETP displayed very weak partial agonism in cAMP accumulation (Fig. 2, E and F) but had no effect on cAMP responses mediated by GLP-1(7–36)NH_2 in interaction assays (Fig. 2E). In the absence of allosteric modulation, the metabolite GLP-1(9–36)NH_2 only exhibited very weak partial agonism for cAMP, with 1000-fold lower potency and only approximately 15% of the maximal signal compared with GLP-1(7–36)NH_2 (Fig. 2F). However, this weak response was strongly potentiated in a concentration-dependent manner by BETP (Fig. 2F). Cooperativity estimates could not be derived for this data set because the operational model of allosterism could not adequately describe the data. Regardless, there is a strikingly strong positive

allosteric effect with both an increase in potency (pEC_{50} shift from 6.4 ± 0.08 to 7.5 ± 0.07) and maximal agonist effect (E_{max} shift from 15 ± 3 to $99 \pm 4\%$) of GLP-1(9–36)NH_2-mediated response (Fig. 2F). For all ligands studied, no response was seen in untransfected cells. Collectively these data identify a novel consequence of allosteric drug action, specifically, the augmentation of metabolite signaling that in two of the three cases studied (the M_2 mAChR and GLP-1R) cannot be predicted from assessment of the parent ligand.

Activation of the GLP-1R by the Major Metabolite of GLP-1(7–36)NH_2 [GLP-1(9–36)NH_2] Is Augmented by Structurally Distinct Small Molecule Allosteric Ligands in a Pathway Selective Manner. To further explore this phenomenon, we performed additional studies using the GLP-1R as a model system. In addition to BETP, we characterized the quinoxaline-based Novo Nordisk compound 2 (Supplemental Fig. 1D) for its ability to modulate the metabolite in cAMP accumulation assays. In a previous study, we showed that compound 2 has a limited ability to augment the actions of GLP-1 or its endogenous peptide variants at the GLP-1R in cAMP signaling, despite showing direct allosteric agonism in its own right (Koole et al., 2010) (Fig. 2G). However, similar to that observed with BETP (Fig. 2, E and F), there was a large potentiation of GLP-1(9–36)NH_2-mediated cAMP signaling (Fig. 2H). Derivation of global cooperativity estimates ($\alpha\beta$) revealed a greater than 400-fold potentiation of the metabolite response and an ~180-fold greater magnitude of positive cooperativity between compound 2 and GLP-1(9–36)NH_2 compared with that for GLP-1(7–36)NH_2 (Table 1). Both compound 2 and BETP exhibited almost neutral cooperativity with GLP-1(7–36)NH_2 and GLP-1(9–36)NH_2 peptides in whole-cell competition binding assays (Supplemental Fig. 4), indicating that the allosteric effects of these compounds on GLP-1(9–36)NH_2-mediated cAMP signaling are principally driven by changes in orthosteric agonist efficacy.

Despite the critical role of GLP-1R-mediated cAMP production in insulin secretion, there is also a role for other signaling components/pathways such as β-arrestin signaling, mobilization of intracellular Ca^{2+}, and activation of mitogen-activated kinases such as ERK1/2 in the augmentation of the insulin response and β-cell survival (Baggio and Drucker, 2007; Sonoda et al., 2008). We therefore extended the study to explore allosteric effects of BETP and compound 2 on GLP-1(9–36)NH_2 in ERK1/2 phosphorylation and Ca^{2+} mobilization (Fig. 3) and compared the effects with the parent peptide. In agreement with our previous findings, GLP-1(7–36)NH_2 displayed robust agonism in ERK phosphorylation and Ca^{2+} mobilization (Fig. 3, A, C, E, and G). Of significance, GLP-1(9–36)NH_2 also displayed agonism in pERK1/2 in a concentration-dependent manner, but only a very weak Ca^{2+} response was observed (at 3 μM peptide). In interaction studies, BETP exhibited negative cooperativity with GLP-1(7–36)NH_2 in ERK phosphorylation but an augmentation in Ca^{2+} signaling at the highest concentration tested (30 μM), with a small increase in pEC_{50} and E_{max} (Fig. 3, A and C; Table 1). In contrast, GLP-1(9–36)NH_2 displayed neutral cooperativity with BETP in ERK1/2 phosphorylation (Fig. 3B), and there was also no apparent change in Ca^{2+} response mediated by GLP-1(9–36)NH_2 in the presence of 30 μM BETP (the small change in response can be attributed to

agonism from BETP alone) (Fig. 3D). Compound 2 displayed neutral cooperativity in both ERK1/2 phosphorylation and intracellular Ca^{2+} mobilization when interacted with either GLP-1(7–36)NH_2 or GLP-1(9–36)NH_2 (Fig. 3, E–H; Table 1). Taken together, these results suggest that allosteric modulation can engender functional selectivity in the actions of both the metabolite and the parent ligand when acting at the GLP-1R. However, the differential modulation between the metabolite and the cognate ligand on the different signaling pathways highlights a novel use of allosteric ligands to engender pathway-selective modulation of response of the metabolite, even if no modulation is observed from the cognate agonist of the system.

Allosteric Modulation of the Metabolite GLP-1(9–36)NH_2 via the GLP-1R Results in Glucose-Dependent Insulin Secretion Ex Vivo in Rat Islets and In Vivo. Activation of the GLP-1R by GLP-1 only increases insulin secretion in conditions of elevated glucose (Göke et al., 1993; Sloop et al., 2010). To evaluate the ability of the metabolite to activate glucose-dependent insulin secretion, pancreatic islets isolated from Sprague-Dawley rats were used. In a previous study, we showed that GLP-1(7–36)NH_2 had insulinotropic activity in islet experiments using high-glucose conditions, and BETP also caused a robust concentration-dependent increase in insulin secretion (Sloop et al., 2010). Here we show that in high glucose conditions, GLP-1(9–36)NH_2 does not induce insulin secretion at concentrations of up to 10 μM (Fig. 4; Supplemental Fig. 5A). However, in the presence of 1 μM BETP (which only minimally increases insulin levels by itself), a dose-dependent increase in GLP-1(9–36)NH_2-mediated insulin-secretion was observed, with a pEC_{50} of 7.4 ± 0.3 (EC_{50} 38 nM) and a maximal response achieved at 100 nM (Fig. 4A). Maximum insulin levels in islet cultures treated with the combination of BETP and 1 μM metabolite were similar to those induced by 100 nM GLP-1(7–36)NH_2 (Supplemental Fig. 5A).

To explore the in vivo insulinotropic effects, glucose-stimulated insulin secretion was measured in compound-treated male Wistar rats undergoing an IVGTT. Similar to our previous study (Sloop et al., 2010), GLP-1(7–36)NH_2 displayed insulin secretagogue activity during the 20-min time course; however, compared with vehicle, animals dosed with 150 nmol/kg GLP-1(9–36)NH_2 had lower levels of plasma insulin than those treated with GLP-1(7–36)NH_2 (Fig. 4B). BETP had no insulinotropic activity at the dose administered (Fig. 4B). However, coadministration of GLP-1(9–36)NH_2 and BETP elicited an elevation in plasma insulin similar to that of animals dosed with GLP-1(7–36)NH_2, although insulin levels remained elevated over the 20-min time period for GLP-1(7–36)NH_2, whereas in the animals dosed with BETP and GLP-1(9–36)NH_2 plasma insulin levels dropped to the level of vehicle after 10 min (Fig. 4B). Determination of total GLP-1 levels throughout the time course showed that GLP-1(9–36)NH_2 was cleared from the plasma within this same 10-min time period and, in addition, revealed that BETP did not alter the pharmacokinetics of GLP-1(9–36)NH_2 (Supplemental Fig. 6). In addition, plasma insulin levels remained elevated [similar to GLP-1(7–36)NH_2] when animals were administered with higher doses of GLP-1(9–36)NH_2 (400 nmol/kg) in the presence of BETP (10 mg/kg) (Supplemental Fig. 5B). Taken together, the ex vivo and in vitro studies support a model whereby BETP allosterically potentiates
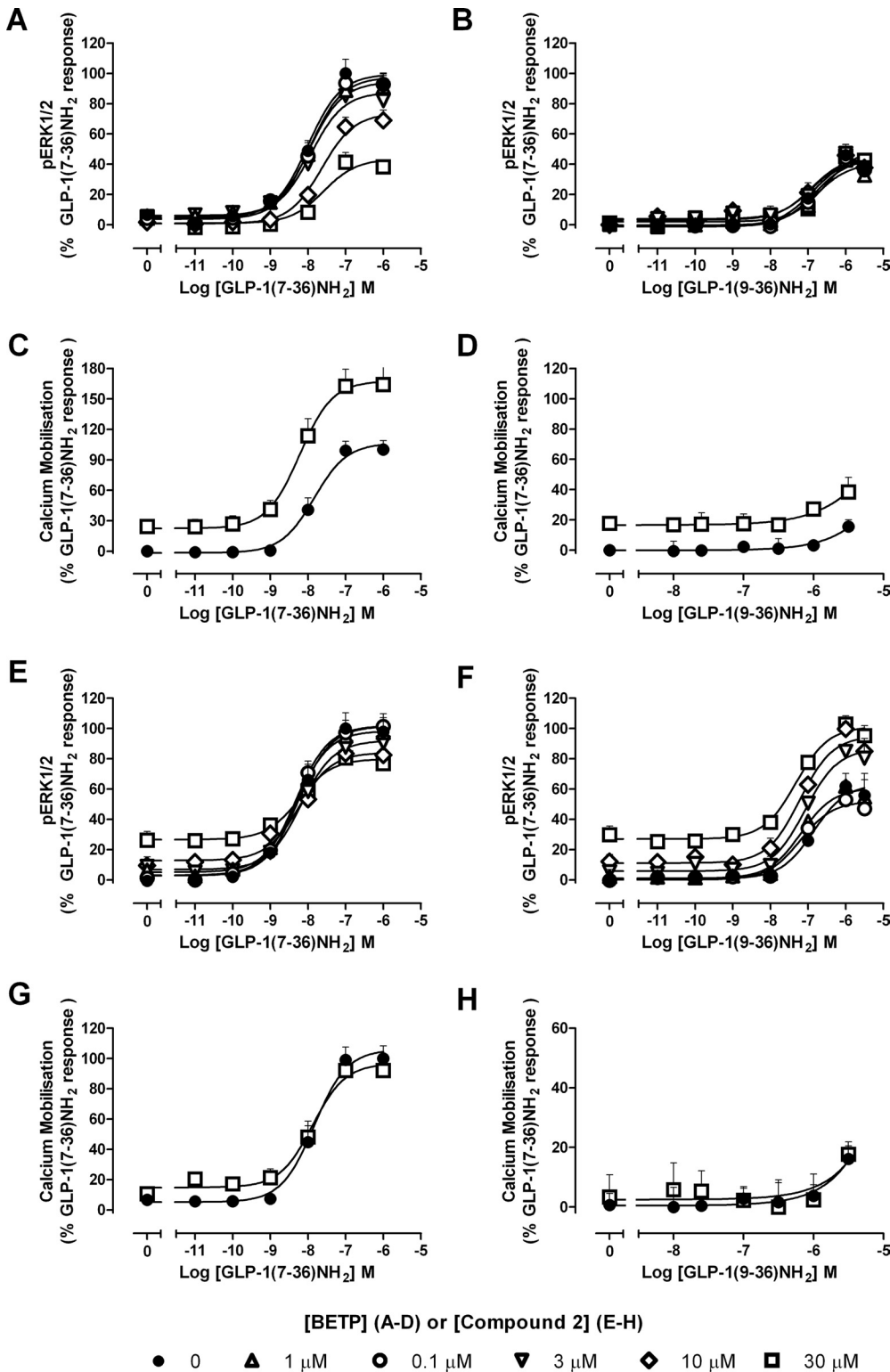
Fig. 3. Differing degrees of allosteric modulation of GLP-1(7–36)NH_2 and GLP-1(9–36)NH_2 by BETP or compound 2 at the GLP-1R in ERK1/2 phosphorylation and intracellular calcium mobilization in intact cells. Interaction studies between BETP (A–D) and GLP-1(7–36)NH_2 (A and C) or GLP-1(9–36)NH_2 (B and D) in ERK1/2 phosphorylation (A and B) or intracellular calcium mobilization (C and D), respectively. Interaction studies between compound 2 (E–H) and GLP-1(7–36)NH_2 (E and G) or GLP-1(9–36)NH_2 (F and H) in ERK1/2 phosphorylation (E and F) or intracellular calcium mobilization (G and H), respectively. All values are means ± S.E.M. of three to four independent experiments performed in duplicate.

GLP-1(9–36)NH_2-mediated cAMP signaling resulting in insulin release. The ability of BETP to specifically augment GLP-1(9–36)NH_2-mediated cAMP signaling in GLP-1R-expressing cells, in combination with the ability to modulate glucose-dependent insulin secretion, provides compelling proof of concept that allosteric potentiation of metabolites is a viable approach for the development of GLP-1R-based therapeutics.

Discussion

In this study, we demonstrate the ability of allosteric ligands to modulate signaling mediated by an inactive metabolite of the primary endogenous ligand. To determine the generality of this hypothesis, three receptors (the GLP-1R, M_2 mAChR, and the A_1-AR) from two different subclasses of GPCRs were selected, each of which is a thera-
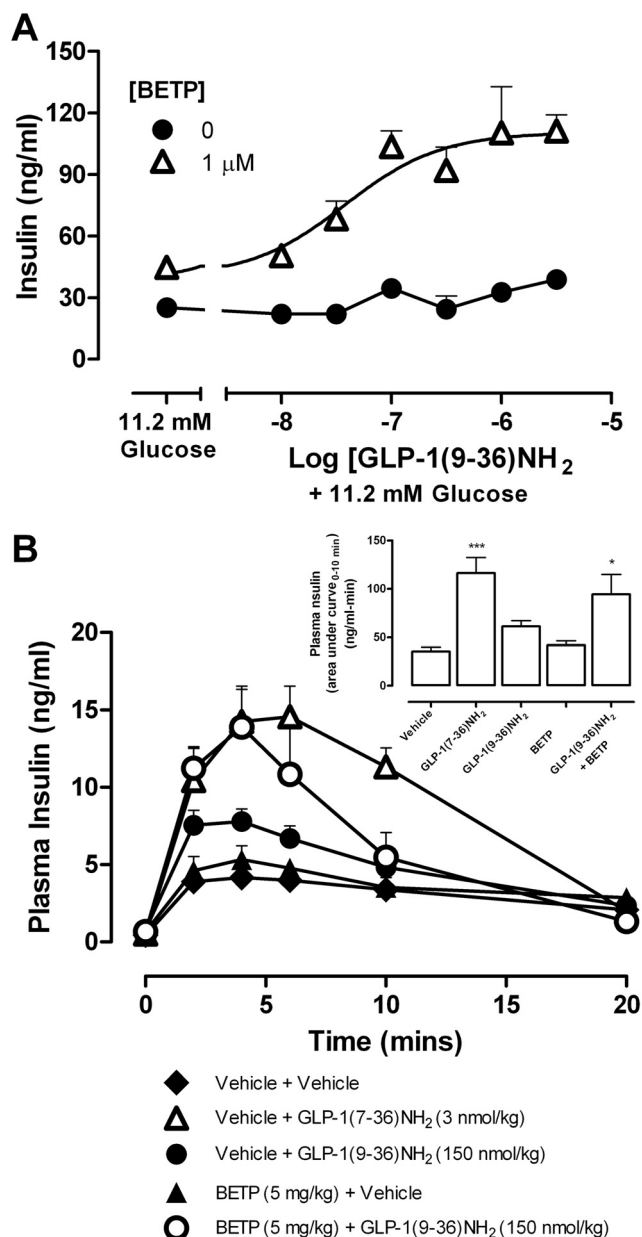
Fig. 4. Ex vivo and in vivo studies reveal allosteric modulation of the GLP-1 metabolite at the GLP-1R leads to insulin secretion. A, insulin concentrations from cultures of Sprague-Dawley rat islets incubated in medium containing high glucose (11.2 mM) and BETP with increasing concentrations of GLP-1(9–36)NH$_2$. Islet treatments were performed for 90 min. B, time course of plasma insulin concentrations in fasted, anesthetized animals treated with either vehicle, GLP-1(7–36)NH$_2$ (3 nmol/kg), GLP-1(9–36)NH$_2$ (150 nmol/kg), BETP (5 mg/kg), or coadministration of BETP and GLP-1(9–36)NH$_2$, immediately before intravenous administration of a glucose bolus (0.5 g/kg). Inset, AUC$_{0-10 min}$ of the insulin secretion for the various treatment groups. All results are expressed as mean ± S.E.M. of five experiments. ∗, $p < 0.05$ as determined using a one-way analysis of variance followed by Dunnett's comparison to vehicle group.

peutic target and also has identified allosteric modulators. The GLP-1R is a family B GPCR and is a promising target in the development of treatments for type 2 diabetes mellitus (DM). Both the M$_2$ mAChR and A$_1$-AR are prototypical family A GPCRs, with separate mechanisms of activation compared with family B GPCRs, and are therapeutic targets for treatment of Alzheimer's disease/asthma and neuropathic pain, respectively.

For all three receptors, the potency of the metabolite alone at the selected signaling pathway (ERK1/2 phosphorylation for Gα$_i$-coupled M$_2$ mAChR and A$_1$-AR and cAMP for Gα$_s$-coupled GLP-1R) was markedly lower than that of the cognate agonist (greater than 1000-fold). However, in each case, an allosteric ligand markedly potentiated signaling by the metabolite. In two of the cases (the GLP-1R and M$_2$ mAChR), the allosteric effect on the metabolite was much more pronounced than the effect on the parent ligand. Taken together, these findings highlight the virtually untapped potential for metabolic products of GPCR endogenous agonists to recruit signaling pathways that would otherwise remain quiescent after inactivation of the parent agonist. The ability to activate responses from convergent and divergent signaling cascades could therefore have the potential to generate a more tuneable response from the metabolite than that of the parent compound.

The ability of each of these allosteric ligands to promote strong potentiation on the actions of the respective metabolite may be therapeutically relevant. Choline levels in the brain have been reported to range between 10 and 15 μM, depending on the species (Tucek, 1985). Choline affinity for the M$_2$ mAChR is low (in the millimolar range); however, both affinity (>10-fold) and potency (>200-fold) can be enhanced by the allosteric ligand LY2033298. This result suggests that it is very likely that allosteric potentiation of these responses may be possible in a physiological setting. Likewise, resting inosine levels in the brain and the heart can reach concentrations as high as 10 μM and at least 30-fold higher in ischemic conditions (Bäckström et al., 2003). Evidence for modulation of inosine at the A$_1$-AR (in addition to previous evidence for modulation at the A$_3$-AR (Gao et al., 2011) provides additional proof that targeting metabolites is viable. Furthermore, circulating GLP-1(9–36)NH$_2$ concentrations are >10-fold higher than that of GLP-1(7–36)NH$_2$ (Göke et al., 1993). However, this metabolite exhibits a ∼1000-fold lower binding affinity for the GLP-1R and equally low efficacy and potency for cAMP accumulation. This observation indicates that at least 100-fold potentiation of the metabolite response would be required for a therapeutically beneficial effect. The in vitro experiments show that compound 2 can produce this degree of potentiation (>250-fold), consistent with modulation of metabolites as a therapeutically relevant approach.

ACh and adenosine both act at several subtypes of the mAChR and adenosine receptor, respectively. Therefore, it is plausible that the metabolites investigated in this study could also have effects at these other subtypes. Certainly this is true for inosine, for which allosteric potentiation of cAMP signaling at the A$_3$-AR has been reported (Gao et al., 2011). One advantage of allosteric ligands is their ability to provide selectivity, and, therefore, use of a selective modulator should, in theory, only modulate the metabolite at the subtype where the allosteric ligand binds.

As an extension of our initial screen, the GLP-1R was used as a model system to further explore the phenomenon. The GLP-1R has actions that address key symptoms associated with DM, including glucose-dependent increases in insulin synthesis and release, decreases in β-cell apoptosis, body mass, and gastric emptying (Vahl and D'Alessio, 2004; Drucker and Nauck, 2006). GLP-1 is principally released from intestinal L cells in its amidated form [GLP-1(7–

36)NH$_2$] in response to meal ingestion, resulting in insulin release (Drucker, 2006). It is very rapidly degraded by dipeptidyl peptidase IV (within 1–2 min) to GLP-1(9–36)NH$_2$, with only ~10% reaching the systemic circulation and even lower levels reaching the pancreatic β cells (Deacon et al., 1995). The metabolite is thus the major circulating form of GLP-1; however, it does not stimulate insulin secretion (Deacon et al., 1995; Tomas and Habener, 2010), presumably due to the lack of GLP-1R-mediated increases in cAMP, which is thought to be a major contributor to insulin secretion.

A novel treatment for type 2 DM, therefore, would be to potentiate the actions of GLP-1(9–36)NH$_2$ mediated through cAMP to elicit insulin secretion. Our results show that this is indeed possible, with two structurally distinct allosteric ligands, BETP and compound 2, able to strongly potentiate cAMP signaling in heterologous cell systems. A key finding in our study was the demonstration that one of these compounds, BETP, could also strongly potentiate the ability of the GLP-1 metabolite to promote insulin secretion in both ex vivo and in vivo rat models. Relatively high concentrations of GLP-1(9–36)NH$_2$ were required to elicit an insulin response (even in the presence of BETP) in the isolated islets (30 nM and above) compared with circulating levels of GLP-1(9–36)NH$_2$ in normal physiology (approximately 100 pM). However, it is not uncommon to require much larger doses of hormones in ex vivo experiments compared with in vivo, for example, the EC$_{50}$ for GLP-1(7–36)NH$_2$ stimulation of islet culture insulin release is 1 to 10 nM (Göke et al., 1993; Sloop et al., 2010; Tomas et al., 2010), only ~10-fold lower than the augmented metabolite response. As the metabolite circulates at >10-fold higher concentrations than the parental peptide, these data suggest that regulation of physiological levels of GLP-1(9–36)NH$_2$ may be possible, even with compounds that have not been optimized for allosteric activity. Further exploration of this phenomenon in vivo at physiological levels of metabolite is currently limited because of the poor pharmacokinetic properties of the allosteric ligands available. However, in vivo effects on insulin secretion and blood glucose elicited by GLP-1(9–36)NH$_2$ are modulated by BETP, albeit with pharmacological dosing with the metabolite. This result provides the proof of concept that modulation of metabolites is possible for physiologically relevant endpoints. It is likely that specific screening programs to identify modulators optimized for potentiation of metabolites are required to conclusively show that allosteric modulation of metabolites can occur in an endogenous system.

To date, the level of modulation seen with the metabolites for existing compounds is purely serendipitous, however, the ability to develop allosteric ligands that induce very strong potentiation certainly exists. Screening programs using the endogenous ligand ACh identified an M$_4$ mAChR allosteric ligand that enhanced the response mediated by ACh 780-fold (Leach et al., 2010). Likewise, benzylquinolone carboxylic acid, an M$_1$ mAChR allosteric ligand, can potentiate the actions of ACh by up to 10,000-fold (Canals et al., 2012). Thus, there is clear precedent for the ability to develop compounds that will be effective even where metabolite activity is only 1/1000th that of the parent ligand (assuming that the metabolite levels do not reach levels higher than those of the parent). Thus, these data provide compelling evidence for proof of concept that allosteric modulation of metabolites

could lead to physiologically relevant responses that are therapeutically beneficial.

At present, for the therapeutically relevant effects of GLP-1R activation, the underlying signaling is not fully understood, but it is clear that physiological responses are a composite of multiple pathways. In our in vitro assays, we showed that allosteric ligands can engender functional selectivity in the actions of the metabolite when acting at the GLP-1R whereby cAMP signaling was strongly potentiated but no change was observed in ERK phosphorylation or Ca^{2+} mobilization. Together with the islet experiments and in vivo studies, this suggests that modulation of cAMP without altering pERK1/2 and calcium signaling is sufficient to promote insulin secretion. Nonetheless, the ideal signaling profiles for other therapeutically relevant effects of GLP-1R activation, such as β-cell survival, still remain to be determined. As more information becomes available, a more detailed understanding of the required combination of collateral efficacies required to therapeutically target different disease states will become apparent. Therefore, information characterizing functional selectivity of all classes of ligands and behavior will become increasingly important in drug discovery programs.

Probe dependence of allosteric drugs has multiple implications in drug discovery and the ability to modulate the action of normally inactive endogenous metabolites could be exploited to develop novel therapeutic agents. In addition, metabolites are often further metabolized, offering additional scope for drug discovery. However, in some cases, modulation of metabolites could also contribute to unwanted or unanticipated side effects of drugs. This study thus highlights the need to understand allosteric effects on all ligands, including metabolites normally considered to be inactive as part of the profile of modulator action. This concept is also relevant for other non-GPCR drug targets, such as ligand-gated ion channels. As a further layer of complexity, the breakdown product of one ligand could activate a different receptor with desirable properties, offering the potential to develop allosteric ligands with properties for modulating that specific receptor target. The findings of pronounced potentiation (in some cases) compared with the endogenous agonist has substantial, previously unrecognized, implications for therapeutic development of small molecule modulators.

Authorship Contributions

Participated in research design: Wootten, Sloop, Willard, Christopoulos, and Sexton.

Conducted experiments: Wootten, Savage, Valant, May, Ficorilli, and Showalter.

Contributed new reagents or analytic tools: Sloop and Willard.

Performed data analysis: Wootten, Savage, Valant, May, Sloop, Willard, Christopoulos, and Sexton.

Wrote or contributed to the writing of the manuscript: Wootten, Sloop, Christopoulos, and Sexton.

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