Exploring Aspects of Allosteric Modulation and Bias Signalling at the Glucagon-Like Peptide-1 Receptor

Emilia Elizabeth Savage
Bachelor Behavioural Neuroscience (Hons)

Submitted in total fulfillment of the requirements of the degree of Doctor of Philosophy

February 2015

Monash Institute of Pharmaceutical Sciences

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ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is an important regulator of insulin biosynthesis and secretion, and is one of the key therapeutic targets in the management of type II diabetes mellitus and obesity. Like most GPCRs, the GLP-1R is pleiotropically coupled, to physiologically relevant signalling pathways including cAMP formation, intracellular calcium ($_{i}Ca^{2+}$) mobilization and phosphorylation of extracellular signal regulated kinases 1 and 2 (pERK1/2).

The GLP-1R is a class B G protein-coupled receptor (GPCR) that has the ability to be activated by multiple endogenous ligands including four variants of GLP-1 (the predominant form being GLP-1(7-36)NH2) and oxyntomodulin. This receptor is also activated by the exogenous peptide exendin-4 and allosteric ligands such as the Novo Nordisk Compound 2 and Eli Lily 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine (BETP). These allosteric ligands also have unique properties compared to orthosteric exogenous ligands including the ability to alter the signalling of the GLP-1R in response to orthosteric ligands. These effects can be different depending on which orthosteric ligand is co bound to the receptor, a effect known as probe-dependence. This thesis identifies a novel case of probe dependence, the ability of allosteric ligands to modify the signalling mediated by metabolites of endogenous ligands that were previously considered to be 'inert' breakdown products that may open up new avenues for allosteric drug discovery.

It is widely accepted that insulin secretion downstream of GLP-1R activation is

critically dependent on cAMP formation, but recent evidence is also emerging for an essential role of regulatory proteins such as β-arrestins and G protein-coupled receptor kinases (GRK). The canonical role of these regulatory proteins is to terminate GPCR signalling and promote receptor internalization. However, more recently, roles as scaffolding proteins that can regulate G protein-independent signalling have emerged. Consequently, the studies comprising this thesis illustrate distinct recruitment profiles of regulatory proteins to the GLP-1R in response to multiple endogenous and exogenous ligands. This thesis identifies differential actions of allosteric modulators on GLP-1R peptide ligands ('probe dependence'), thus demonstrating differential responses of receptor signalling with respect to both orthosteric and allosteric ligands, highlighting the ability for both ligand- and pathway-specific effects ('biased signalling'). Collectively, this work further demonstrates the potential benefits of biased signalling and allosteric modulation, but may also influence the approaches and precautions that must be considered in the design, identification and development of small molecules for therapeutic use.

DECLARATION

In accordance with Monash University Doctorate Regulation 17/Doctor of Philosophy and Master of Philosophy (MPhil) regulations, the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any other university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

This thesis includes two original manuscripts published in peer-reviewed journals. The core theme of the thesis is "Modulation of regulatory signalling pathways at the human glucagon-like peptide-1 receptor." The ideas, development and writing of all papers in this thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmacology under the supervision of Prof. Patrick Sexton and Dr. Denise Wootten.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

ACKNOWLEDGEMENTS

To begin, I would like to thank my principal supervisors Dr. Denise Wootten and Prof. Patrick Sexton for offering me the opportunity to complete a PhD in drug discovery biology and welcoming me to the world of family B GPCRs at Monash Institute of Pharmaceutical Sciences. Their guidance, insight and generosity have made my candidature a truly rewarding experience.

To Denise Wootten, you planted the seed well in advance, even before I had finished my honours year, where you proposed the idea of me partaking in a PhD under you. I will never forget that afternoon's lighthearted conversation in the bacteria lab, where I went from feeling overwhelmed and having self-doubt in my abilities to feeling valued and motivated to finish. I had someone who believed in me and was committed to my greatness as a scientist. Your unlimited support, guidance and patience has been humbling, and has made me the scientist I am today. I feel that with the skills I have learnt from you throughout my PhD, including the ability to communicate and present professionally to a large audience, troubleshoot, problem solve, write, think independently, teach others, and the capability to network are all valuable skills that are transferable to life. I would like to thank you for your kindness and all that you have contributed towards this PhD, I will be forever grateful.

A huge thank you to past and present members of the Drug Discovery Biology laboratory (DDBL) and Department of Pharmacology in particular to my little buddy Cass and to Jon, Mo mo, Tinamarie, Adriel and Sheng. During those days of continuing unsuccessful experiments and despair, at times when I struggled to pick myself up and start all over again, it was my DDBL buddies that encouraged, inspired and motivated me. I have established many wonderful friendships in DDBL, all of which I cherish, they have been there every step of the way though out this long challenging journey. With these special friends we have shared wisdom, guidance, joy, and food. The times spent in the tearoom drinking coffee and chatting about whatever topic was something I looked forward to every day. I appreciate all their time and patience spent proof reading my work.

Finally, I am forever grateful of the support from my friends and above all my family. They have been my incredible “A” team and rock. In particular I want to thank my fiancé Michael, for his endless encouragement and belief in me. He always had a way of making me smile and convinced me that food was the cure for everything. He instilled balance in my life, and always made sure I was on my toes trying and doing different things. Regardless of my work load he kept my mind, body and soul at peace and made sure I always knew he was there for me. Finally, I want to thank my sister Jacqui, for spending multiple days helping and teaching how to use Adobe Illustrator so that I could transform my colorful childlike diagrams into clear and publishable figures.

In the case of Chapter 2-3, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and Extent of Candidate's Contribution
2	Allosteric Modulation Of Endogenous Metabolites As An Avenue For Drug Discovery	Published	Development of ideas, performed experiments and data analysis, contribution to writing and revision of manuscript (50%)
3	A simple method to generate stable cell lines for the analysis of transient protein-protein interactions	Published	Development of ideas, performed experiments and data analysis, contribution to writing and revision of manuscript (50%)

Signed:  _ Date: __05/02/2015

PUBLICATIONS AND COMMUNICATIONS

Peer-Reviewed Articles

Savage EE, Wootten D, Christopoulos A, Sexton P and Furness SG. (2013). *A simple method to generate stable cell lines for the analysis of transient protein-protein interactions*
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*Co-first author

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Investigating Recruitment Of Regulatory Proteins To The Glp-1r And Their Role In Downstream Signalling. Poster presentation. Gordon Research Conference/Seminar. April 2013. Lucca (Barga), Tuscany Italy.

Emilia E Savage, Denise Wootten, Arthur Christopoulos and Patrick M Sexton. *Characterization Of Glucagon-Like Peptide-1 Receptor Interaction With B-Arrestins And G Protein-Coupled Receptor Kinases.* Poster presentation. Keystone Symposia. February 2012. Banff, Canada.

E Savage, C Valant, A Christopoulos, Drug Discovery Biology Laboratory, Monash University, Victoria, Australia 3052. *Investigation Of Stimulus Bias Of Orthosteric And Allosteric Ligands Of The Human A_1 Receptor In Yeast Saccharomyces Cerevisiae.* Poster presentation. GPCR WORKSHOP. December 2010. Honolulu, Hawaii.

Emilia E Savage, Denise Wootten, Arthur Christopoulos and Patrick M Sexton. *Allosteric Modulation Of Regulatory Protein Recruitment To The Glucagon-Like Peptide-1 Receptor.* Poster presentation. ASCEPT Annual Scientific Meeting. December 2012. Sydney, Australia.

Emilia Savage, Denise Wootten, John Simms, Cassandra Koole, Celine Valant, Arthur Christopoulos and Patrick M Sexton. *Ligand Directed Bias Signalling At The Glucagon Like-Peptide 1 Receptor (Glp-1R)*. Poster presentation. Monash Obesity And Diabetes Institute (modi^{TM}) Workshop. December 2011. Melbourne, Australia.

Emilia Savage, Celine Valant, Greg Stewart, Patrick M Sexton, Peter J Scammells and Arthur Christopoulos, *Novel Allosteric Modulators and Hybrid Orthosteric/Allosteric Ligands of the Adenosine A1 Receptor* Poster presentation. ASCEPT Annual Scientific Meeting. November 2010. Melbourne, Australia.

Invited Seminars

Emilia E Savage, Denise Wootten, Arthur Christopoulos and Patrick M Sexton. *Ligand Directed Bias at the Glucagon Like-Peptide 1 Receptor (GLP-1R)*. May 2013. SCRIPPS Research Institute Florida, USA.

Emilia E Savage, Denise Wootten, Arthur Christopoulos and Patrick M Sexton. *Investigating recruitment of regulatory proteins to the GLP-1R and their role in downstream signalling*. October 2013. 1st Annual Drug Discovery Biology Student Symposium, Monash University, Melbourne, Australia.

Emilia E Savage, Denise Wootten, Arthur Christopoulos and Patrick M Sexton. *Characterization of glucagon-like peptide-1 receptor interaction with β-arrestins and G protein-coupled receptor kinases*. November 2012. 7th Annual Postgraduate Research Symposium, Monash University, Melbourne, Australia.

ABBREVIATIONS

5-HT – 5-hydroxytryptamine AA – arachidonic acid
AC – adenylyl cyclase
ACh - acetylcholine
AM – acetoxymethyl
AP-2 – activating protein 2
β-Arrestin – beta arrestin
ANOVA – analysis of variance
ARF6 – ADP-ribosylation factor 6
ARNO – ARF nucleotide exchange factor
ATCM – allosteric ternary complex model
ATP – adenosine triphosphate
ATSM – allosteric two-state model
BCA – bicinchoninic acid
BETP – 4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine
Boc5 - 1,3-bis [[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid
Bpa – p-benzoylphenylalanine
BRET - bioluminescence resonance energy transfer
BSA – bovine serum albumin
Ca^{2+} – calcium
cAMP – cyclic adenosine monophosphate
CaS – Ca^{2+} sensing
CHO – Chinese hamster ovary
CCP – clathrin coated pits
Compound 1 – 2-(2'-methyl)thiadiazolylsulfanyl-3-trifluoromethyl-6,7-dichloroquinoxaline
Compound 2 – 6,7-dichloro2-methylsulfonyl-3-tert-butylaminoquinoxaline
CREB – cAMP response element-binding
CRF – corticotropin-releasing factor
c-Src – proto-oncogene tyrosine kinase Src
DAG – diacylglycerol
DM – diabetes mellitus
DMEM – Dulbecco's modified eagle medium
DOI – (6)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DPPIV – dipeptidyl peptidase IV
ECL – extracellular loop
EGF – epidermal growth factor
Epac – exchange protein activated by cAMP
ERK1/2 – extracellular signal regulated kinases 1 and 2
FBS – fetal bovine serum
GDP – guanosine diphosphate
GHRH – growth hormone-releasing hormone
GIP – glucose-dependent insulinotropic polypeptide, gastric inhibitory polypeptide
GLP – glucagon-like peptide
GLP-1R – glucagon-like peptide 1 receptor
GPCR – G protein-coupled receptor

G protein – guanine nucleotide-binding protein
GPS – GPCR proteolytic site
GRK – G protein receptor kinase
GRPP – glicentin-related polypeptide
GSK – glucogen synthase kinase
GTP – guanosine triphosphate
HIV – human immunodeficiency virus
HTS – high throughput screen
iCa^{2+} - intracellular Ca^{2+}
IBMX – 3-isobutyl-1-methylxanthine ICL – intracellular loop
InP – intervening peptide
IP – inositol phosphate
IRS – insulin receptor substrate
IUPHAR – International Union of Basic and Clinical Pharmacology
JNK – c-Jun N-terminal kinases
K$^+$ – potassium
LY2033298 – 3-amino-5-chloro-6-methoxy-4-methyl-thieno(2,3-b)pyridine-2-carboxylic acid cyclopropylamide
mACh – muscarinic acetylcholine
MAPK – mitogen-activated protein kinase
McN-A-343 – 4-(m-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride
MEK – mitogen-activated protein kinase kinase
MPGF – major proglucagon fragment
Na$^+$ – sodium
NAM – negative allosteric modulator
NEP – neutral endopeptidase 24.11
NFxviB – nuclear factor kappa B
NMR – nuclear magnetic resonance
NMS – N-methylscopolamine
NSF – N-ethylmaleimide-sensitive fusion
OPD – o-phenylenediamine dihydrochloride
ORG – Organon research
PAC – PACAP receptor
PACAP – pituitary adenylate cyclase-activating peptide
PAM – positive allosteric modulator
PBS – phosphate buffered saline
PC – prohormone convertase
PDB – Protein Data Bank
PEG – polyethylene glycol
pERK1/2 – phosphorylated ERK1/2
PI3K – phosphatidylinositol-3 kinase
PIP2 – phosphatidylinositol 4,5-bisphosphate
PFA – paraformaldehyde
PLC – phospholipase C
PLD – phospholipase D
PKA – protein kinase A
PKB – protein kinase B
PKC – protein kinase C
PTH – parathyroid hormone

PTx – pertussis toxin
QNB – quinuclidinyl benzilate
Quercetin – 3,3',4,5,7-pentahydroxyflavone
Raf – mitogen-activated protein kinase kinase kinase
RAMP – receptor activity modifying protein
RhoA – Ras homolog gene family member A
Forskolin - (3R,4aR,5S,6S,6aS,10S,10aR,10bS)-6,10, 10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-3- vinyldodecahydro-1H-benzo[f]chromen-5-yl acetate
SAR – structure activity relationship
SCR – short consensus repeat
SNP – single nucleotide polymorphism
SOCS – suppressor of cytokine signaling proteins
SR141716A – N-(piperidin- 1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride
SU – sulfonylurea
TM – transmembrane
TT15 - (2*S*)-2-[[(8*S*)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid
TZD – thiazolidinedione
VDCC – voltage-dependent Ca2+ channel
VIP – vasoactive intestinal peptide
VPAC – VIP and PACAP receptors
VU0029767 – (E)-2-(4-ethoxyphenylamino)-N'-((2-hydroxynaphthalen-1-yl)methylene)acetohydrazide
ZDF – Zucker diabetic fatty

CHAPTER 1:

General Introduction

Section 1:

1.1 G protein-coupled receptors (GPCRs)

1.1.1 General introduction

G protein–coupled receptors (GPCRs) are the largest class of membrane spanning proteins identified in the human genome and they regulate many biological processes (Lagerström & Schiöth 2008). GPCRs transmit information from extracellular stimuli to intracellular signals, eliciting multiple cellular responses that play essential roles in human health and disease. GPCRs can interact with a large variety of ligands from small biogenic amines to large glycoprotein hormones and they are the target of approximately 30% of all clinically prescribed drugs (Lagerström & Schiöth 2008; Ahrén 2009; Drews 2000; Hopkins & Groom 2002; Overington et al. 2006).

1.1.2 Structural characteristics of GPCRs

GPCRs share low overall sequence homology, however they share a high degree of structural homology. They are composed of seven alpha (α)-helical hydrophobic transmembrane (TM) domains (TMs 1-7), connected by three alternating intracellular (ICLs 1-3) loops and three extracellular loops (ECLs 1-3), an extracellular amino-terminus and an intracellular carboxyl-terminal domain (Baldwin 1993). These helices show a clockwise arrangement when observed from the extracellular side (Wess 1997). The majority of GPCRs also possess two conserved cysteine residues; one in ECL2 and the other at the top of TM3 that forms a disulphide bond that is important for stability and structural integrity (Bockaert & Pin 1999).

1.1.3 Classification of GPCRs

GPCRs are classified into three major receptor subclasses A, B and C, on the basis of their sequence homology. Family A (or rhodopsin- like GPCRs; otherwise termed Class I or Class A receptors) is the largest (approximately 90 % of all GPCRs) and most extensively studied family and includes receptors for visual rhodopsin, biogenic amines and olfactory stimuli receptors, among others (Lagerström & Schiöth 2008; Palczewski et al. 2000). This subfamily of GPCRs contains a series of highly conserved amino acid motifs and is the only family for which there are high-resolution crystal structures of the full length GPCR. Family B GPCRs (otherwise termed Class II or Class B receptors) comprise approximately 48 members, and their predominant feature is a large extracellular N-terminus (approximately 150 residues) (Parthier et al. 2007), that is the principle binding site for their endogenous ligands (Yona et al. 2008). Family C (otherwise termed Class III or Class C) are another small class of GPCRs, comprising metabotropic glutamate receptors, the $GABA_B$ receptor, the calcium sensory receptor (CaSR) and some taste receptors (Pierce et al. 2002; Breitwieser et al. 2004). These function as obligate dimers containing a large N-terminal globular domain often referred to as the 'venus fly trap domain' that is the primary binding site for endogenous ligands.

1.2 Family B (Adhesion and Secretin) GPCRs

1.2.1 General Introduction

The Family B subfamily is the second largest family of GPCRs and can be further subdivided into two groups (adhesion and secretin). Of the mammalian receptors in this family there are 33 adhesion and 15 secretin-like members (Fredriksson et al. 2003). These receptors are recognized by their highly glycosylated long extracellular N-terminus that preferentially binds extracellular

molecules and peptides. The adhesion receptors possess a distinct GPCR proteolytic (GPS) domain that is important for accurate folding and trafficking of the receptor to the cell membrane, with multiple complex functional domains. Receptors of this family can be subdivided into eight subgroups I-VIII, some of which include; brain–specific angiogenesis-inhibitory, lectomedin receptors and epidermal growth factor (EGF)-like module containing receptors (Bjarnadóttir et al. 2005; Krasnoperov et al. 1997; Lagerström & Schiöth 2008).

1.2.2 Family B/Secretin-like GPCRs

The secretin-like family B GPCRs includes calcitonin and calcitonin-like receptors (CALCR, CALCRL); corticotropin-releasing factor receptors (CRFR1, CRFR2); the glucagon receptor (GCGR); the gastric inhibitory polypeptide receptor (GIPR); the glucagon-like peptide receptors (GLP-1R, GLP2R); the growth-hormone-releasing hormone receptor (GHRHR); the adenylate cyclase activating polypeptide receptor (PAC1/ADCYAP1R1); the parathyroid hormone receptors (PTHR1, PTHR2); the secretin receptor (SCTR) and the vasoactive intestinal peptide receptors (VPAC1R/VPAC2R), these are reviewed in (Lagerström & Schiöth 2008). Secretin-like family B GPCRs each possess long N-termini, with a network of conserved cysteine residues that form three disulphide bridges that are essential for correct folding of this domain and binding of large endogenous peptide hormones. (Hofmann et al. 2001; Grauschopf et al. 2000; Bazarsuren et al. 2002). These receptors have enormous potential as therapeutic targets for future drug development. Calcitonin, glucagon and parathyroid hormone are examples of three clinically used peptides for the treatment of hypercalcaemia, hypoglycaemia and osteoporosis respectively. Additional therapeutic potential for these receptors as drug targets includes VPAC1R and PACR for neurodegenerative disorders (Brenneman 2007) and inflammation (Abad et al. 2006), GLP-1R, GCGR and amylin receptors for type 2 diabetes mellitus (T2DM), GLP-2R for bowel disorders (Hornby & B. A. Moore 2011), CRFR are for chronic stress (Zoumakis et al. 2006), PTHR (O'Brien et al. 2008) and CTRs (Pondel 2000) for bone disorders

and CGRP receptors for migraine and pain (Russo 2015). For the remainder of this thesis, reference to family B GPCRs will refer to just the secretin subfamily of GPCRs.

1.2.3 Structural information on family B GPCRs

The past decade has witnessed a pronounced increase in structural biology techniques that have allowed the determination of high-resolution crystal structures of GPCRs, however to date structures are only available for family A GPCRs. Structural knowledge for family B GPCRs is limited, however, recent studies have solved crystal structures of the isolated TM bundle of two family B GPCRs. These include a 3.0-ångström (Å)-resolution structure of the CRF1R in complex with a small-molecule inhibitor (Hollenstein et al. 2013) and a 3.4-Å-resolution structure of the GCGR using a version of the TM domain of the protein that was largely unmodified (Siu et al. 2013). In addition to these TM domain structures, there are crystal and nuclear magnetic resonance (NMR) structures of the N-termini of many family B receptors including GIPR (Parthier et al. 2007), CRF1R and CRF2R (Pioszak & H. E. Xu 2008; C. R. Grace et al. 2004; C. R. R. Grace et al. 2010; C. R. R. Grace et al. 2007), PTH1R (Pioszak & H. E. Xu 2008) and GLP-1R (Runge et al. 2008; Underwood et al. 2010). A common feature shared between these N-terminal domains is a 'sushi domain' or 'short consensus repeat' (SCR), characterised by two antiparallel β-sheets that comprise the sushi domain as well as disulfides and a salt bridge (C. R. Grace et al. 2004; C. R. R. Grace et al. 2007; Grauschopf et al. 2000; Parthier et al. 2007; PERRIN 2006; Pioszak & H. E. Xu 2008; Runge et al. 2008). These crystal structures have been solved in complex with peptide ligands and reveal that, although individual residues that interact with ligands vary between the different subtypes, the binding pocket for these ligands within the N terminal domain is highly conserved.

1.3 GPCR Signalling

1.3.1 GPCR G protein-dependent signalling

Activation of GPCRs classically results in G protein-dependent signalling that involves coupling of the receptor with heterotrimeric G proteins. G proteins are GTPases that consist of three subunits α, β, and γ (Milligan & Kostenis 2006). There are multiple alpha (α), beta (β), and gamma (γ) subunits and although not all combinations are favoured, this multiplicity allows for diversity in signalling.

Inactive G proteins exist as heterotrimers (Gαβγ). Upon activation of a GPCR via either ligand-induced activation or constitutive activity of the receptor alone, conformational rearrangements occur in the receptor that lead to the exposure of intracellular binding pockets/sites for G proteins. Interaction of the GPCR with the G protein promotes catalytic exchange of GDP for GTP on the α subunit, resulting in dissociation of Gα from Gβγ, although there is evidence that in some cases the catalytic exchange results in a rearrangement of the subunits, but that they remain associated (Smrcka 2008). Following GDP/GTP exchange, these subunits can then activate or inhibit several effector proteins, as well causing additional physiological changes due to influences on ion channel function.

Distinct classes of Gα proteins have been identified that couple to GPCRs and they primarily differ in the amino acid sequence of their N-terminal portion (that forms interactions with the receptor). Principally these have been classified into four groups $G\alpha_s$, $G\alpha_{i/o,}$ $G\alpha_{q/11}$ and $G\alpha_{12/13}$ each of which is responsible for regulating specific cellular functions (Neves et al. 2002) (Figure 1.1). $G\alpha_s$ proteins couple to adenylate cyclase (AC) causing catalytic conversion of ATP to cyclic AMP (cAMP), and subsequent activation of protein kinase A (PKA) and the exchange proteins directly activated by cAMP (EPAC) family of cAMP-regulated guanine nucleotide

exchange factors, both of which influence multiple downstream effectors. In contrast, $G\alpha_{i/o}$ proteins inhibit the function of AC. $G\alpha_{q/11}$ proteins activate phospholipase C (PLC-β) signalling to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers calcium (Ca^{2+}) mobilization from the endoplasmic reticulum (ER) whereas DAG activates protein kinase C (PKC) (Winzell & Ahrén 2007). $G\alpha_{12/13}$ activate Rho guanine-nucleotide exchange factors (GEFs), promoting cytoskeletal rearrangement and changes in membrane structure. In addition, Gβγ subunits can regulate intracellular signalling pathways (Figure 1.1). There are five Gβ-subunits ($G\beta_1$-$G\beta_5$), and 12 Gγ-subunits ($G\gamma_1$-$G\gamma_{12}$), grouped according to minor deviations in amino acid sequence of the protein. Gβγ dimers are involved in phosphorylation of extracellular signal-related kinase 1 and 2 (pERK1/2), activation of PLC and phosphatidylinositol 3' (PI3K), regulation of potassium (K^+) and Ca^{2+} channels and recruitment of G-protein receptor kinases (GRK2/3) (Lagerström & Schiöth 2008; Smrcka 2008; Khan et al. 2013).

1.3.2 Regulation of GPCRs

Several mechanisms exist to regulate the length and strength of GPCR signals. Including RGS proteins that can have profound effects on the duration, magnitude and concentration dependence of GPCR signals. The exposure of a GPCR to an agonist often results in the rapid attenuation of receptor responsiveness. This process, known as desensitisation, can occur at the level of the receptor, the G protein or the effector system. At the level of the GPCR, the molecular mechanisms that govern desensitisation include uncoupling the receptor from the G proteins, internalisation of cell surface receptors to intracellular compartments and downregulation of total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as degradation of preexisting receptors. The timeframes over which these processes occur range from seconds (uncoupling) to minutes (endocytosis) to hours (downregulation) and the extent of desensitisation varies from complete removal of signal to attenuation of agonist potency and efficacy (NJ & RJ 1996; Lefkowitz & Whalen 2004; Pierce &

Lefkowitz 2001).

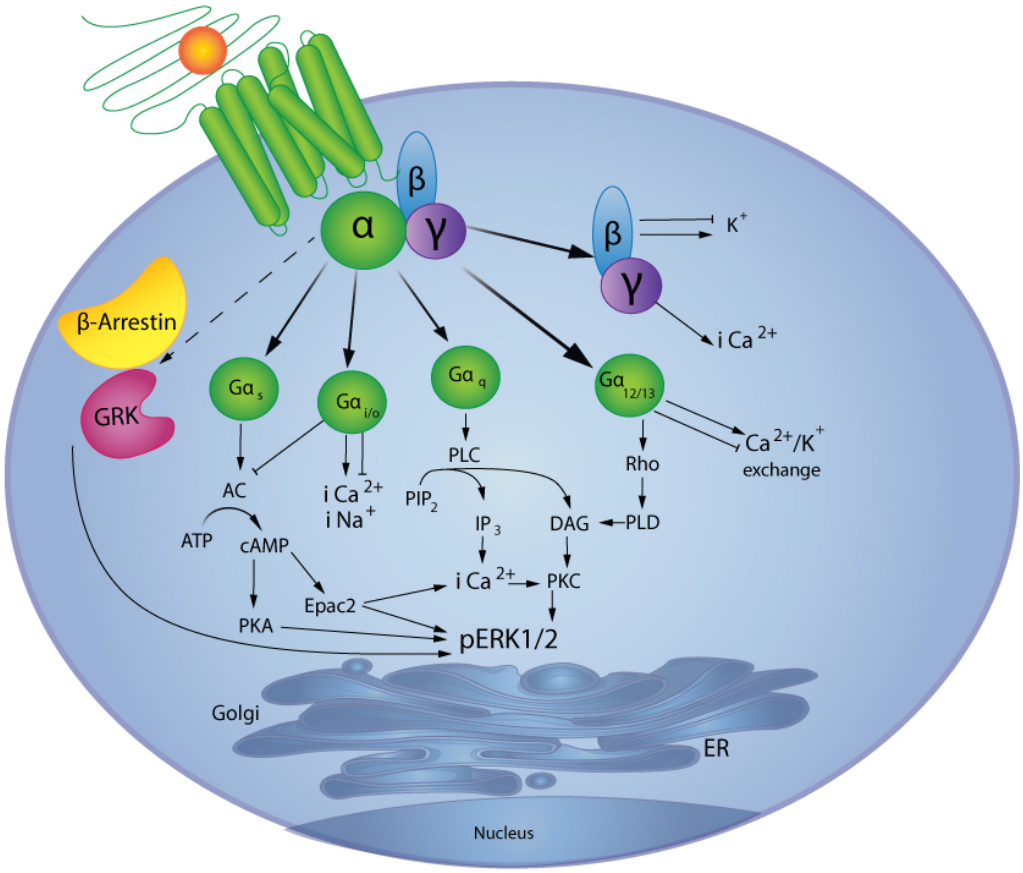


Figure 1.1 Major G protein-mediated signalling pathways

Exchange of GDP for GTP on the α-subunits of the G protein complex allows dissociation of the α subunits. G proteins are responsible for receptor activation, receptor desensitisation and regulation of various signalling cascades, the major of which are detailed here. For Gαs proteins, these include increases in intracellular cAMP accumulation, activation of protein kinase A (PKA) and exchange protein activated by cAMP-2 (Epac2). For Gαi/o proteins, these include regulation of ${}_{i}Ca^{2+}$ and intracellular sodium (${}_{i}Na^{+}$) levels and inhibition of adenylate cyclase, while Gαq stimulates PLC activation as well as phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol triphosphate (IP3) and diacylglycerol (DAG), each of which leads to increases in ${}_{i}Ca^{2+}$ mobilization and protein kinase C (PKC) and subsequent pERK1/2. For $G\alpha_{12/13}$ proteins, these include regulation of Ca^{2+}/potassium (K^{+}) and increases in Rho, stress fiber formation, and phospholipase D (PLD). For the Gβγ-complexes, these include regulation of ${}_{i}Ca^{2+}$ and ${}_{i}K^{+}$ recruitment of β-arrestins and GRKs, and like most other pathways, contribute to regulation of mitogen-activated protein kinase (MAPK) signalling cascades, which includes the phosphorylation of extracellular signal regulated kinases 1 and 2 (pERK1/2).

Receptor uncoupling from G proteins occurs as a result of receptor phosphorylation by intracellular kinases that include second messenger kinases (eg protein kinase A (PKA) and protein kinase C (PKC)) and G protein-coupled receptor kinases (GRKs) (Figure 1.2). Receptor phosphorylation causes high affinity arrestin binding (desensitisation), thus, uncoupling the receptor from the G protein in many cases directing the receptor to clathrin coated pits (CCPs) through the binding of endocytic elements AP-2, clathrin, ARF6, ARNO and NSF promoting receptor internalization (C. A. C. Moore et al. 2007; Marchese et al. 2003; Krupnick & Benovic 1998). β-arrestins also bind and are ubiquitinated by the E3 ubiquitin ligase Mdm2 (Shenoy et al. 2001). This agonist-stimulated ubiquitination event is required for β-arrestin–mediated endocytosis, but precisely how or why is not known. Following endocytosis, receptors undergo degradation in lysosomes or are recycled back to the cell membrane (Lefkowitz & Shenoy 2005).

1.3.3 Regulation and non-canonical signalling by GPCRs

Previously, the only known role of GRKs and β-arrestins was as terminators of G protein-mediated signalling through their roles in GPCR desensitisation, internalisation and recycling (Pierce & Lefkowitz 2001; Shukla et al. 2011). Emerging evidence presents a new paradigm of non-canonical (G protein-independent) signalling, typically occurring through β-arrestins, whereby β-arrestin coupling can lead to direct activation of signalling pathways independent of G protein activation (Lefkowitz & Shenoy 2005) (Figure 1.3). Furthermore, stabilization of distinct receptor conformations by distinct ligand/receptor combinations is proposed to result in specific phosphorylation patterns on the intracellular face of the receptor that involve distinct GRKs (Xiao et al. 2010) . The phosphorylation pattern has been speculated to act as a “barcode” (Tobin 2009; Tobin et al. 2008), directing β-arrestin conformational changes, thus controlling

their interaction partners and subsequent downstream signalling roles and related functions (Liggett 2011; Reiter et al. 2012; S. Rajagopal et al. 2010). β-arrestins therefore act as

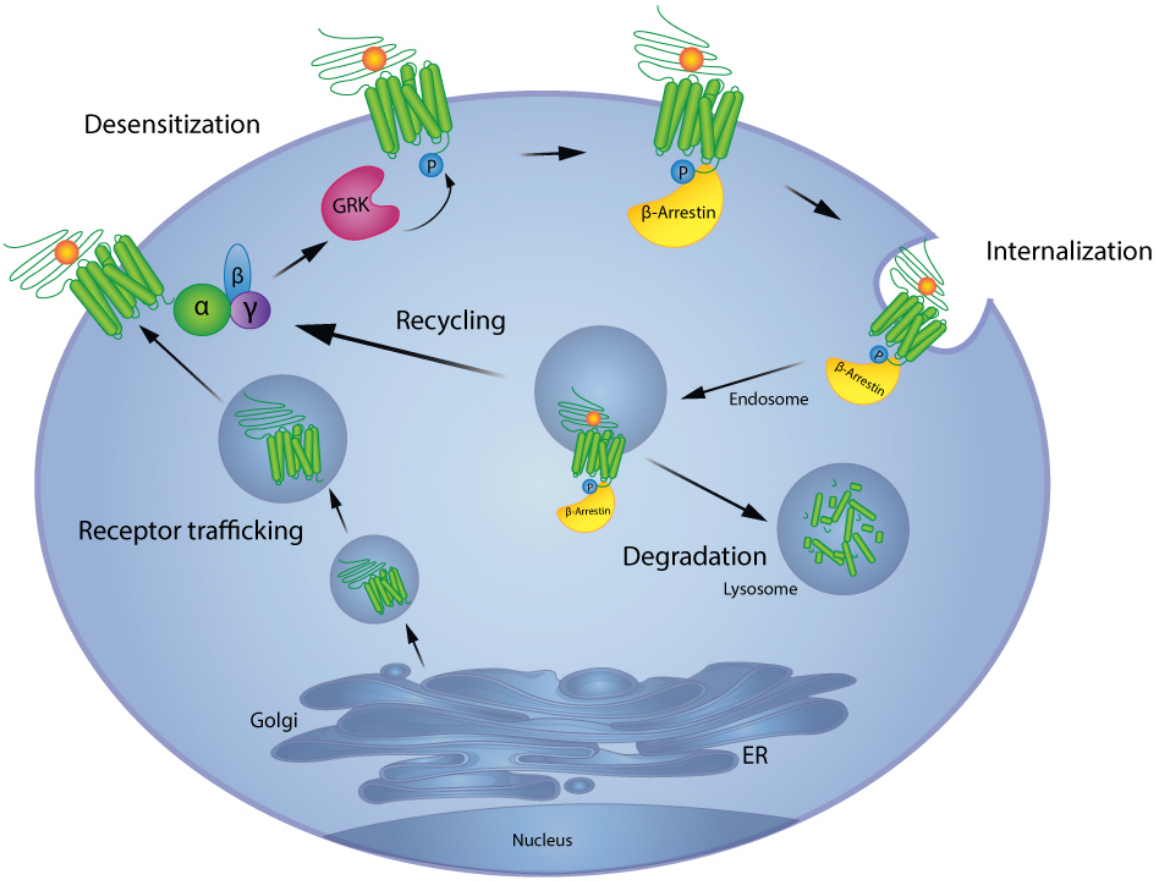


Figure 1.2 Schematic of canonical GPCR regulation by GRKs and β-arrestins

The classical view of GPCR regulation begins by activation of the receptor, followed by GRK phosphorylation of its C-terminus, resulting in a higher affinity for arrestins to bind. Binding of β-arrestin terminates G protein signalling and targets the receptor to clathrin coated pits where the receptor is either internalised and recycled back to the cell membrane or sentenced to degradation in lysosomes.

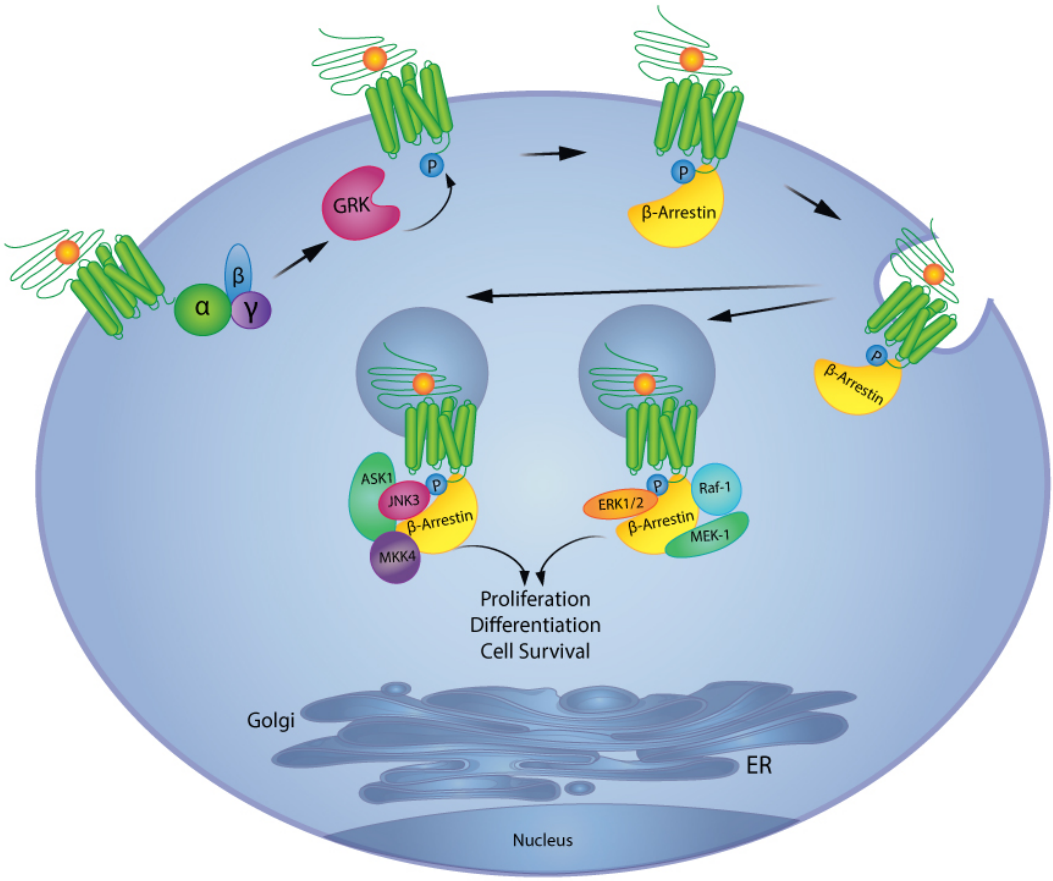


Figure 1.3 Schematic of non-canonical GPCR regulation by GRKs and β-arrestins

Non-canonical GPCR regulation can lead to the formation of arrestin-dependent signalsomes that can affect a diverse number of cellular processes. Independent of G protein activation, arrestins recruit effector enzymes that are able to promote the degradation of second messengers and regulate GPCR endocytosis and intracellular trafficking, complementing their classic roles in receptor desensitization. This schematic highlights arrestin-based signalling complexes and how they contribute to cytosolic processes such as cell proliferation, differentiation and survival.

multifunctional scaffolds interacting with many different proteins and tyrosine kinases causing phosphorylation of numerous intracellular targets (Lagerström & Schiöth 2008; Heuss & Gerber 2000; Ahrén 2009; Drews 2000; Hopkins & Groom 2002; Overington et al. 2006) (Bockaert & Pin 1999; Baldwin 1993; S. Rajagopal et al. 2010; Wess 1997; Wisler et al. 2014).

1.3.4 β-arrestins

As briefly outlined earlier, β-arrestins are multifunctional pleiotropic adaptor proteins, typically known to terminate heterotrimeric G protein signalling. Upon recruitment to an activated receptor they can terminate G-protein mediated signalling and promote internalisation by directing the receptor to clathrin-mediated endocytosis (Lagerström & Schiöth 2008; Pal et al. 2013; Palczewski et al. 2000; Lin & DeFea 2013), but they can also activate downstream signalling cascades in their own right.

Following identification of visual arrestins came the discovery of two β-arrestin (β-arrestin1 and β-arrestin2) spliced iosoforms that share 80% amino acid sequence homology. The cDNA for human β-arrestin variants differ only in the absence of 24 base pairs/8 amino acids within the C-terminal region (Parthier et al. 2007; Parruti et al. 1993; Lefkowitz 2013a). Multi-protein complexes are a common feature of β-arrestin mediated signalling. The first well characterised complex identified was the mitogen-activated kinase modules associated with (ERK1/2) and JNK3. Multiple other kinases have since been implicated in β-arrestin-dependent signalling, some associated with positive and some negative physiological outcomes. These include Raf, MEK1, Ask1, MKK4, Akt, phosphatidylinositol-3 kinase (PI3K) (Yona et al. 2008; DeFea 2011), Lim-domain-containing kinase (LIMK), calcium/calmodulin kinase II (CAMKII), calcium/calmodulin kinase kinase β(CAMKKβ) and adenosine monophosphate-activated protein

kinase (AMPK) (Pierce et al. 2002; DeFea 2011; Breitwieser et al. 2004). The mechanisms by which β-arrestins promote the activation of these cascades and the composition of individual MAPK module/β-arrestin complexes varies between receptors, however a full understanding of the complexity of this signalling has not been established. Studies on the β_2-adrenergic receptor (β_2AR) revealed that there was a β-arrestin-dependent component mediating ERK1/2 phosphorylation that involves scaffolding and activation of Src (Fredriksson et al. 2003; Luttrell et al. 1999). Other receptors also display a similar profile of β-arrestin-mediated ERK1/2, including protease-activated receptor-2 (PAR2) (Bjarnadóttir et al. 2005; DeFea et al. 2000; Krasnoperov et al. 1997; Lagerström & Schiöth 2008), and the angiotensin II 1A receptor (AT1aR) (Lagerström & Schiöth 2008; Tohgo et al. 2002). Both PAR2 and AT1R signal activation via β-arrestins-mediated pERK1/2, activation whereas NK1R requires Gαq-coupled signalling events for β-arrestin recruitment while PAR2R involves β-arrestin-dependent membrane sequested ERK1/2.

The identification and understanding of β-arrestin dependent signalling is expanding rapidly. β-arrestin2 is critically involved in CXCR4-mediated chemotaxis, mediated by its enhancement of p38 MAPK activation. This has major implications for lymphocyte homing, hematopoiesis and breast cancer metastasis (Hofmann et al. 2001; Sun et al. 2002; Grauschopf et al. 2000; Bazarsuren et al. 2002). Moreover, the virally encoded GPCR US28 is constitutively phosphorylated and recruits β-arrestin in the absence of agonist (Brenneman 2007; W. E. Miller et al. 2003). There is evidence to suggest β-arrestins are capable of directly interacting with IκBα inhibitor of (NFκB), a key molecule in innate and adaptive immunity that might prevent the phosphorylation and degradation of NFκB. Consequently, β-arrestins effectively modulate activation of NFκB and expression of NFκB target genes, presenting themselves as important regulators in the immune systems (Abad et al. 2006; H. Gao et al. 2004; Witherow et al. 2004). β-arrestin 2 also functions as a mediator of kinase/phosphatase scaffolding of Akt (protein kinase

B) and PP2A, which is responsible for the regulation of Akt by dopamine receptors (DAR). This presents β-arrestin 2 as a positive mediator of dopaminergic synaptic transmission and a potential pharmacological target for dopamine-related psychiatric disorders (Hornby & B. A. Moore 2011; Beaulieu et al. 2005). In addition, upon activation of $AT_{1A}R$, β-arrestin 1 stimulates the small guanosine triphosphate Ras homolog gene family member A (GTPase RhoA), leading to the re-organization of stress fibres that is a fundamental process required for cell motility, adhesion and contraction (Zoumakis et al. 2006; Barnes et al. 2005; Hollenstein et al. 2013; Reiter & Lefkowitz 2006a).

1.3.5 G protein-coupled receptor kinases

G protein-coupled receptor kinases (GRKs) belong to the protein kinase A, G, and C (AGC) family. GRKs phosphorylate serine/threonine residues located within the C-terminal tail and/or the ICLs of activated GPCRs, allowing for high affinity binding of β-arrestin1 and/or β-arrestin2 (arrestin2 and arrestin3) as previously discussed (Siu et al. 2013; Willets, Challiss & Nahorski 2003a).

There are seven members of the GRK family (GRK1-7), however only GRK2, GRK3 GRK5 and GRK6 are ubiquitously expressed in mammalian tissue. Expression of mammalian GRK1 and GRK7 is largely limited to vertebrate rod and cone photoreceptors, whereas GRK4 is highly expressed in the testis (Milligan & Kostenis 2006; E. V. Gurevich et al. 2011; Shenoy & Lefkowitz 2011). All GRKs are multi-domain proteins that possess a similar structural organization with an N-terminal domain (~185 amino acids) unique to the GRK family of kinases, followed by the regulator of G protein signalling (RGS) homology domain (RH) (Neves et al. 2002; Siderovski et al. 1996), a highly conserved Ser/Thr protein kinase domain (KD) and a C-terminal domain (Winzell & Ahrén 2007; Métayé et al. 2005). The C-termini of the GRKs contain structural elements responsible for their membrane targeting, and contribute to their

subcellular localization by favoring their interaction with lipids and other membrane protein. The N-terminal region is important for receptor recognition and intracellular membrane anchoring (Carman, Parent, et al. 1999b), the RH domain is involved in receptor binding and the catalytic domain mediates substrate phosphorylation (NJ & RJ 1996; Willets & Challiss 2003; Lefkowitz & Whalen 2004; Ross & Wilkie 2000; Pierce & Lefkowitz 2001; Pao & Benovic 2002). Structural organization of GRKs is based on sequence similarity and gene structure such that GRK1 and GRK7 comprise of a short C-terminal prenylation sequence allowing the addition of hydrophobic molecules and thereby facilitating attachment to the cell membrane, whereas GRK2/3 contain a pleckstrin homology (PH) domain that interacts with G proteins βγ (Gβγ) subunits: (C. A. C. Moore et al. 2007; Pitcher et al. 1992; Marchese et al. 2003; Koch et al. 1993; Krupnick & Benovic 1998; DebBurman et al. 1996; Carman et al. 2000). GRK4 and GRK6 contain palmitoylation sites (Shenoy et al. 2001; Stoffel et al. 1994; Premont et al. 1996), along with positively charged elements (Lefkowitz & Shenoy 2005; Jiang et al. 2007) that allow them to bind to lipids, whereas GRK5 relies solely on positively charged lipid-binding elements for lipid association (Pierce & Lefkowitz 2001; Pitcher et al. 1996; Shukla et al. 2011; Thiyagarajan et al. 2004; E. V. Gurevich et al. 2011). Due to these features GRKs 1, 4, 5, 6 and 7, are membrane associated where as GRK2 and 3 are cytosolic.

In order for GRKs to phosphorylate agonist-bound receptors, they must initially form a complex with intracellular domains of the receptor, primarily the C-terminus. GRKs 1, 4, 5, 6, and 7 are positioned at the membrane, neighboring the activated receptors that they bind and phosphorylate. Cytosolic GRK2 and 3 undergo transient recruitment to the plasma membrane after receptor activation and bind to PIP2 in the plasma membrane via their PH domains (Lefkowitz & Shenoy 2005; Pitcher et al. 1995). However, translocation of these kinases is dependent on G protein activation, as GRK2 and GRK3 bind Gβγ dimers where they are recruited to the membrane and these are only available after dissociation from Gα subunit (Xiao

et al. 2010; Pitcher et al. 1992). GRK2 and GRK3 can also bind the activated GTP-bound form of the Gαq subunit through domains located near the amino termini of the kinases, domains that show significant homology to the regulator of G protein signalling (RGS) family of proteins. They are therefore able to limit the extent of G_q-coupled receptor signalling by sequestering $G_{\alpha q}$ and preventing its coupling to downstream effectors (Liggett 2011; Kohout & Lefkowitz 2003; Reiter et al. 2012; Berman & Gilman 1998; S. Rajagopal et al. 2010). The RGS domain of GRK2 binds $G\alpha_{q/11}$ but not $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, or $G\alpha_{12/13}$ (Pao & Benovic 2002).

In addition to GRKs, other kinases (such as PKA and PKC that are activated by cAMP and DAG/IP3 respectively) can directly induce receptor desensitisation by phosphorylating GPCRs. More recently, it has been demonstrated that PKA and PKC can also affect GPCR desensitisation by phosphorylating GRK2 and altering its activity. This has been shown following β_2AR stimulation, whereby cellular levels of cyclic AMP increase and PKA becomes activated, resulting in enhanced receptor phosphorylation. PKA phosphorylates GRK2 at serine 685, thereby increasing its binding affinity for $G_{\beta\gamma}$ dimers and thus promoting the recruitment of GRK2 to the plasma membrane and into a complex with its activated receptor substrates (Kohout & Lefkowitz 2003).

In addition to phosphorylation, there is increasing evidence implicating GRKs in phosphorylation-independent desensitisation of GPCRs. Not only do GRK interactions mediate uncoupling of receptor and G protein (desensitization) through GPCR phosphorylation and subsequent recruitment of arrestins, but also through physical association with the receptor, or direct association with and inhibition of $G\alpha_{q/11}$ (Ribas et al. 2007; Pao & Benovic 2002). This has been shown for endothelin A and B receptors (Freedman et al. 1997), thromboxane A2

receptors (Carman, Parent, et al. 1999b), α_{1b} adrenergic receptors (Diviani et al. 1996), M_1 and M_3 muscarinic cholinergic receptors (Carman, Parent, et al. 1999b; Willets et al. 2004), PTHRs (Dicker et al. 1999), thyrotropin-releasing hormone (TRH) receptors (Sallese, Mariggiò, et al. 2000b), 5-hydroxy tryptamine 2C receptors (Sallese, Mariggiò, et al. 2000b), metabotropic glutamate receptor-1a (mGluR1a) (Sterne-Marr et al. 2004; Dale et al. 2000; Dhami et al. 2005), type 1A angiotensin II receptors ($AT_{1a}R$) (Usui et al. 2000) and human H1 histamine receptors (Iwata et al. 2005).

In addition to interacting with and phosphorylating GPCRs, GRKs also interact with other proteins including Gα-proteins (Carman, Parent, et al. 1999b; Sallese, Mariggiò, et al. 2000b), Gβγ (Pitcher et al. 1992; Carman et al. 2000), clathrin (Shiina et al. 2001), GRK-interacting protein (GIT1) (Premont et al. 1998), cavelolin-1 (Carman, Lisanti, et al. 1999a), phosphoionositide 3-kinase-α and γ (Naga Prasad et al. 2001), the cytoskeletal proteins tubulin and actin (Pitcher et al. 1998; Carman et al. 1998; Freeman et al. 1998) and Ca^{2+} binding proteins (Sallese, Iacovelli, et al. 2000a).

GRKs therefore play a crucial function in both GPCR desensitization and other protein regulation, and there are many reports implicating their activity in a range of human diseases, including heart failure and opiate addiction. GRKs may therefore be therapeutic targets for management of some diseases.

1.3.6 GPCRs and biased signalling

GPCRs are promiscuous in that they can pleiotropically couple to multiple G proteins and other intracellular effectors, thus allowing for a plethora of downstream effects via different signalling pathways that are dependent on the bound ligand. It is now acknowledged that not all ligands stimulate the entire repertoire of cellular responses associated with receptor activation (Kenakin 2005). The phenomenon of ligand-directed signalling bias, (also referred to functional

selectivity, ligand bias, biased signalling, or biased agonism), arises due to distinct ligands stabilizing different repertoires of receptor conformations, each of which leads to activation of distinct signal transduction pathways (Kenakin 2005). Biased ligands can selectively stabilize a particular subset of receptor conformations to the exclusion of others, thus promoting selective coupling to different pathways with differing prominence and these can be G protein-dependent or independent (Galandrin et al. 2008). The simplest way to identify biased signalling is by reversal of potency and/or efficacy of ligands in different signalling pathways (Figure 1.4). Biased signalling is evident at multiple GPCRs. The first clear demonstration of this was shown for the PAC1R, where agonist PACAP(1-38) stimulates cAMP with greater potency than agonist PACAP(1-27), conversely PACAP(1-38) is more potent for IP stimulation than PACAP(1-27) (Spengler et al. 1993). Other examples include, the neurokinin 1 receptor (NK-1R), where the agonists [Pro^9]substance-P and septide dsplay similar potencies for IP hydrolysis, but [Pro^9]substance-P potently stimulates cAMP, whereas septide induces only weak coupling to cAMP (Sagan et al. 1996); the serotonin 5HT receptor, where the agonist (6)-1-(2,5-dimethoxy-4- iodophenyl)-2-aminopropane (DOI) favors PLC-mediated IP accumulation with low efficacy for phospholipase A2-mediated arachidonic acid (AA) release, whereas quipazine has low efficacy in IP accumulation and high efficacy in AA release (Berg et al. 1998); the PTH1R, where PTH(1-34) activates both PKA downstream of cAMP and PKC downstream of IP, whereas PTH(1-31) solely activates PKA, and PTH(3-38) solely activates PKC (reviewed by (Luttrell & Kenakin 2011). Furthermore, biased signalling has also been identified at the μ-receptors (Keith et al. 1996), DAR (Urban et al. 2007), V_2 vasopressin receptor (V_2R) (Barak et al. 2001), cannabinoid receptors (Georgieva et al. 2008) and GLP-1R (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a) among others. In particular, there has been growing evidence of ligands that induce β-arrestin biased signalling; examples include the β2AR (Azzi et al. 2003; Drake et al. 2008), $AT_{1a}R$ (H. Wei et al. 2003),

V_2R(Azzi et al. 2003) and PTH1R (Gesty-Palmer, Flannery, Yuan, Corsino, Spurney, Lefkowitz & Luttrell 2009a; Rominger et al. 2014).

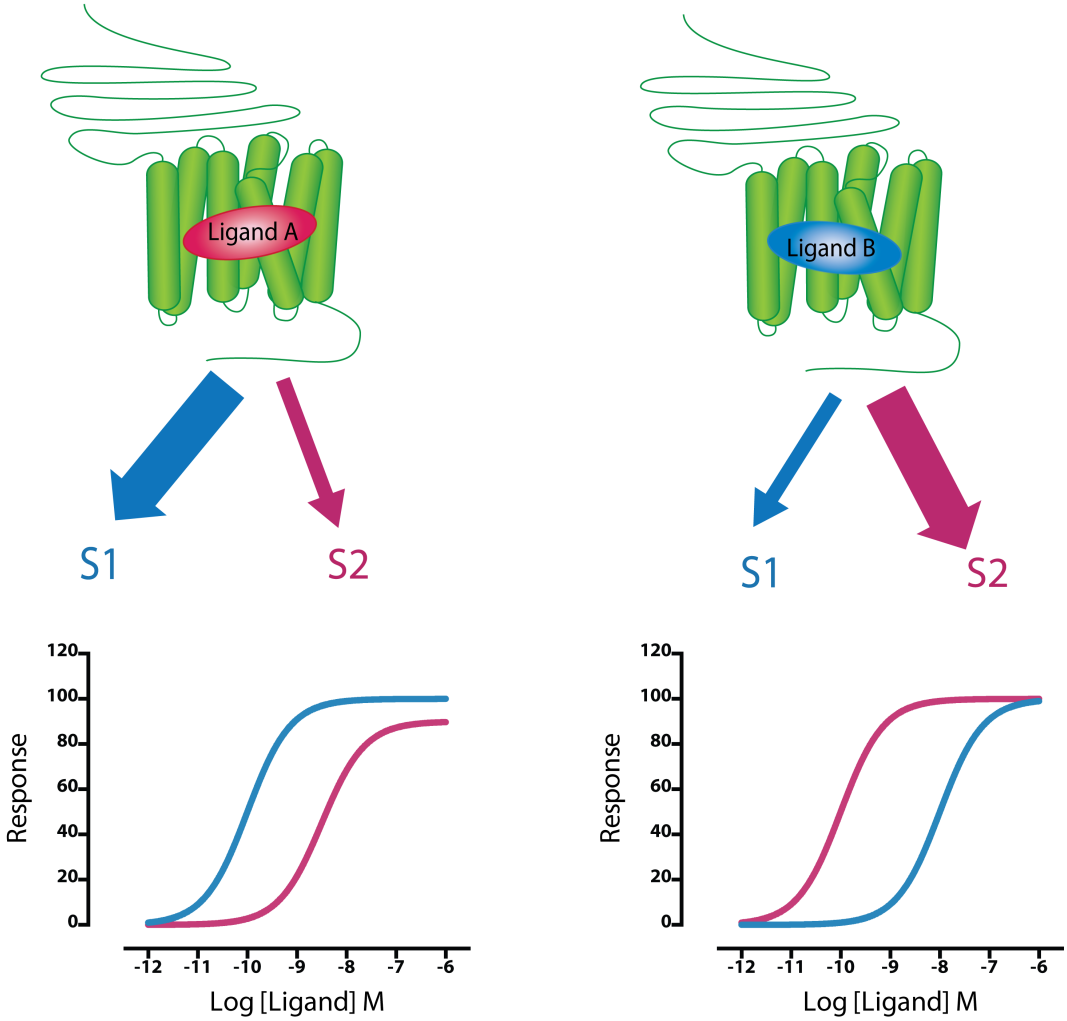


Figure 1.4 Signalling bias between orthosteric ligands

Signalling bias may be identified by reversal of potency and/or efficacy in different signalling pathways by individual ligands. In this example ligand A shows a higher potency for signalling down pathway S1 compared to S2, while binding of ligand B displays a distinct signal bias profile compared to ligand A with a switch in potency between S1 and S2 signalling pathways.

Observations of biased signalling at GPCRs have led to speculation that the optimal mix of efficacies for different signalling pathways will determine the ultimate clinical efficacy of therapeutics. This is exemplified by examples in the literature where ligands that activate β-arrestin-mediated signalling pathways present positive therapeutic effects, whereas G protein–dependent signalling causes unwanted side effect profiles (although this profile is not universally associated with therapeutic benefit). For example, the β-blocker carvedilol acts as a β-arrestin-biased ligand at both β1-adrenergic receptor (β1AR) and β2AR subtypes and provides cardioprotective effects via activation of epidermal growth factor receptor (EGRF) and ERK1/2 phosphorylation in a G protein-independent manner. In contrast, β-AR-mediated activation of G protein-dependent signalling (Gαs coupling) is cardiotoxic (X. Zhang et al. 2013b). In addition, for many years it was not understood why some β-blockers were more effective therapeutics than others as all had the ability to block receptor mediated activation of G proteins. The revelation in the past decade that these more effective "β-blockers" are able to not only block G protein activation via the β2AR, but can also induce β-arrestin mediated signalling, whereas the less effective therapeutics do not signal via β-arrestins, provides strong evidence for the therapeutic relevance of biased signalling for future drug development (Shenoy 2011; van der Westhuizen et al. 2014). Similar to the β2AR, agonists acting at the $AT_{1a}R$ can also display biased signalling with the peptide ligand (TRV120027) unable to active Gαq, but retaining the ability to recruit β-arrestin 2 that results in ERK1/2 phosphorylation as well as receptor internalisation (Violin et al. 2010). *In vivo* studies using this β-arrestin biased ligand have reported a reduction in mean arterial blood pressure and increased cardiac contractility (K. Rajagopal et al. 2006). This provides further evidence that biased ligands have the potential to modulate certain signalling pathways to the exclusion of others that could provide therapeutic advantages (S. Rajagopal et al. 2010). This newly appreciated complexity of GPCR signalling provides the potential for therapeutics that may selectively augment or hinder distinct signalling

pathways to fine-tune receptor signalling, however this phenomenon may also lead to unwanted side effects. One of the key challenges currently in the field is to determine the extent of biased signalling by distinct ligands and also to understand which pathways lead to beneficial physiological effects versus unwanted side effect profiles. Therefore it has become increasingly increasingly relevant to understand the full repertoire of GPCR drug action and of endogenous ligand signalling.

1.4 Allosteric modulation of GPCRs

1.4.1 General Introduction

GPCRs are a valuable class of targets for therapeutic intervention with approximately 30-40 percent of todays approved drugs modulating these proteins but they are yet to be exploited to their full therapeutic potential. Traditionally, drug discovery has focused on targeting the binding site of the natural endogenous ligand for that receptor (the orthosteric site). However, in recent years, targeting binding sites that are topographically distinct from the orthosteric site (allosteric sites) has gained significant attention. Allosteric agonists bind to allosteric sites producing a physiological response in the absence of an endogenous ligand (Figure 1.5). In contrast, allosteric inverse agonists reduce receptor constitutive activity. However, as these ligands bind to topographically distinct sites to orthosteric ligands (Kenakin & L. J. Miller 2010; MONOD et al. 1963), they can bind simultaneously with an orthosteric ligand, causing a change in receptor conformation that engenders a change in the biological activity of the protein (Figure 1.5). The result of this interaction may potentiate or inhibit the binding and/or signalling of an orthosteric ligand (Figure 1.5). Ligands that enhance binding and/or function of the orthosteric ligand are termed positive allosteric modulators (PAMs), those that inhibit binding and/or function are negative allosteric modulators (NAMs), whereas allosteric ligands that bind the receptor, but do

not alter the binding and/or function are referred to as neutral allosteric ligands (NALs)

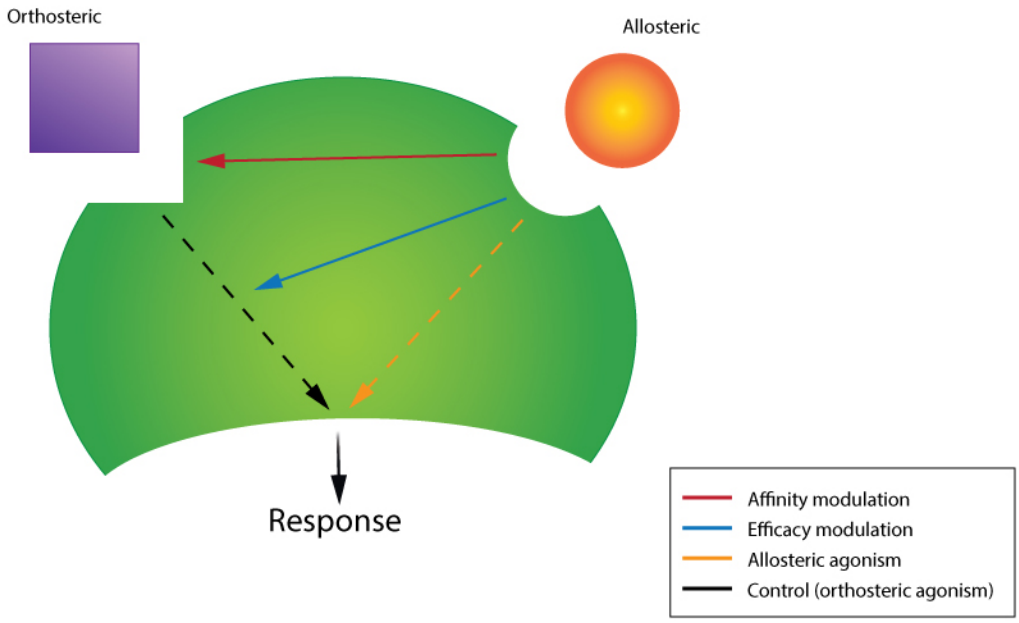


Figure 1.5 Schematic diagram of allosteric actions

Simultaneous interaction of an orthosteric and allosteric ligand at a receptor can have two major effects; affinity modulation, whereby allosteric ligand influences the binding of the orthosteric ligand; and/or efficacy modulation, whereby the allosteric ligand influences the functional output of the orthosteric ligand. In addition, the allosteric ligand may have agonism of its own, independent of orthosteric ligand interaction at the receptor (adapted from Langmead and Christopoulos, 2006).

(Christopoulos et al. 2014). Effects that are engendered between orthosteric and allosteric ligands are described as 'cooperative'.

1.4.2 Therapeutic advantages of allosteric modulators

Until recently, traditional drug therapies for GPCRs have focused on targeting the orthosteric binding site using mimetics (synthetic compounds based on the endogenous ligand). However, the orthosteric binding pocket of related GPCRs is often highly conserved, for example, there are five subtypes of mAChRs, twelve subtypes of 5-HTRs, four subtypes of adenosine receptors, five subtypes of DARs. This therefore provides a major challenge for the development of orthosterically acting therapeutics, due to their poor subtype selectivity, and undesired effects at related receptor subtypes. In comparison to orthosteric sites, topographically distinct allosteric binding sites are not subject to the same evolutionary pressure and as a consequence are less conserved amongst receptor subtypes. Therefore drugs that bind an allosteric site are more likely to achieve subtype selectivity. One well characterised example of an allosteric ligand that displays receptor subtype selectivity is LY2033298 that positively modulates ACh in Ca^{2+} mobilization assays at the M_4 mAChR, but not the other four subtypes of mAChRs (Chan et al. 2008; Yeatman et al. 2014).

Allosteric ligands offer further advantages to orthosteric ligands in that they have a limit to their effect (defined by their cooperativity), beyond which no further modulation can be observed, this is otherwise described as having saturability in their effect and provides target based safety in instances of drug overdose. Furthermore, barbiturates and ethanol are allosteric ligands for GABAa receptors that can produce effects greater than GABA alone. In addition, pure allosteric modulators (that do not display agonism) only have the capacity to modulate in the presence of their associated orthosteric ligand, therefore offering the possibility of fine-tuning existing physiological responses while maintaining the spatial and temporal characteristics of endogenous

signalling. In addition, the allosteric binding site might be amenable to targeting with small molecules on GPCRs where the orthosteric binding site is diffuse and poorly druggable, as is seen for receptors with large peptidic ligands; commonly found with the Family B subclass of GPCRs. Generally, these advantages of allosteric modulators apply regardless of the particular therapeutic area and location of the receptor being targeted.

A crucial part of drug development is understanding of the acute and chronic effects of allosteric modulators at their corresponding targets. In recent years, extraordinary progress has been made in the discovery of allosteric ligands that have different pharmacological profiles, however, despite there being over 100 reported allosteric modulators of GPCRs, only two have gained FDA approval to date (Conn et al. 2009); cinacalcet, a CaSR PAM used for hypercalcemia and hyperparathyroidism (Block et al. 2004; Lindberg et al. 2005), and maraviroc, a CCR5 allosteric antagonist used in HIV-1 treatment (Fätkenheuer et al. 2005; Wood & Armour 2005; Rosario et al. 2008).

1.4.3 Bitopic ligands

Many reported "allosteric" agonists may actually be "bitopic" ligands. These are hybrid orthosteric/allosteric ligands that bind simultaneously to both the orthosteric and allosteric sites within a given GPCR. These bitopic ligands can have several advantages over an allosteric ligand. They can theoretically have improved affinity over pure allosteric ligands, owing to a greater number of ligand-receptor contacts (through binding to both sites), and can also engender selectivity (over orthosteric ligands) due to targeting an allosteric site. Furthermore, while in some diseases when endogenous ligand tone is unaltered and an allosteric ligand would be an advantage, in diseases where there is a reduction in endogenous ligand concentration, bitopic ligands may provide some of the advantages of allosteric ligands (engendering selectivity but with improved affinity/efficacy for a given receptor) but eliminate the potential need for co-

administration of drug treatments (Melancon et al. 2012).

1.5 Allosterism and probe-dependence

1.5.1 General Introduction

Probe-dependence describes the paradigm whereby the extent and direction of an allosteric interaction can vary with the nature of the orthosteric ligand used as a probe of receptor function. Simply, allosteric modulators can produce distinct effects on different orthosteric ligands acting at the same GPCR (Kenakin 2005; Keov et al. 2011; Kenakin & L. J. Miller 2010). For example, the CCR5 allosteric modulator aplaviroc produces very little effect on the binding of the chemokine CCL5 to the receptor but completely blocks the binding of the chemokine CCL3 (Watson etal.2005; Kenakin 2012). Another example of probe dependence involves LY2033298, which positively modulates the binding affinity of the orthosteric agonist, ACh, at the M_4 mAChR, but is essentially neutral when tested against two structurally distinct orthosteric antagonists, [^{3}H]N-methylscopolamine ([^{3}H]NMS) or [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) (Leach et al. 2010). LY2033298 also robustly potentiates pERK1/2 signalling by ACh and oxotremorine, but not xanomeline at the M_4 mAChR (Suratman et al. 2011). Brucine, an allosteric modulator of the M_2 mAChR has shown distinctive modulatory profiles in the presence of various probes as evidenced by their varying cooperativities (α); brucine + McN-A-343 (α=42), brucine + pilocarpine (α=0.86), brucine + ACh (α=3.5), brucine + oxotremorine (α=5.1) (Jakubik et al., 1997; D. S. Kang et al. 2013), whereby these α values are indicative of positive cooperativity. This property of allosteric ligands has implications therapeutically. Probe dependence requires that, wherever possible, the natural endogenous ligand interacting with the receptor be present in the screening milieu to detect physiologically relevant interactions; there is requirement of careful consideration in the choice of orthosteric ligands to assess the effects of an allosteric modulator when the natural ligand cannot be used (Kenakin 2008; Leach et al.

2007).

Careful consideration when pharmacologically characterizing allosteric ligands also applies to receptors possessing multiple endogenous ligands. For example, at the GLP-1R, the allosteric ligand, Compound 2, produces a <5-fold potentiation of cAMP response to the endogenous agonist GLP-1(7-36)NH_2, but a 25-fold potentiation of cAMP by the endogenous agonist oxyntomodulin (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a). These types of studies suggest that all endogenous agonists for a given receptor need to assessed when characterizing the effects of allosteric modulators (Kenakin 2012).

1.5.2 Interplay of allosterism, probe dependence and biased signalling

Classically, allosteric modulation of GPCRs and biased signalling are described as separate phenomena, but both arise from ligand-specific conformational changes in the GPCR. Just as the binding of an orthosteric agonist, or allosteric agonist can give rise to distinct conformational changes that can lead to biased signalling, the co-binding of an orthosteric and allosteric ligand simultaneously can stabilize distinct conformational states of the GPCR compared to the binding of one ligand alone. A consequence is that some pathways may be selectively modulated (either positively or negatively) at the expense of others (Figure 1.6). The most extreme situation of this is where the allosteric effect is in opposite directions for two different pathways (ie positive in one and negative in the other). PDC113.824 (an allosteric inhibitor of parturition) is an example of this. In mouse models, this compound acts as a negative allosteric modulator of prostaglandin F2α receptor-mediated cytosolic and myometrial contraction through uncoupling the receptor from the $G\alpha_{12}$- Rho-ROCK signalling pathway, while significantly augmenting the activation of ERK1/2 via $G\alpha_q$ (Goupil et al. 2010). There are also more subtle examples of signalling bias induced by an allosteric modulator. For example, potentiation of M_4 mAChR signalling by the

allosteric ligand LY2033298 engenders substantial differences in the magnitude of positive cooperativity across several signalling pathways (Nawaratne et al. 2010; Valant et al. 2012). Another clear example is at the M_1 mAChR with VU0029767 (allosteric ligand). VU0029767

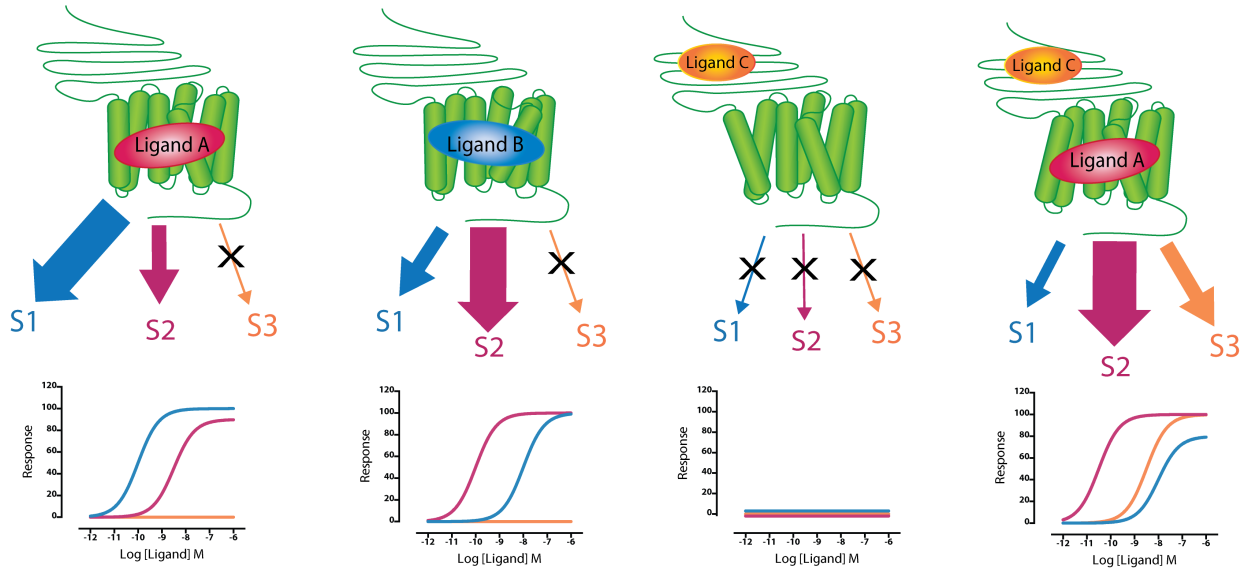


Figure 1.6 Signalling bias by orthosteric agonists, allosteric agonists or by allosteric modulation

Signalling bias may be identified by a reversal of efficacy by different ligands in distinct signalling pathways. In this example orthosteric ligand A shows a higher potency for signalling down pathway S1 compared to S2, but does not signal via pathway S3. Binding of an orthosteric agonist, ligand B, displays a distinct signal bias profile compared to ligand A with a switch in potency between S1 and S2 signalling pathways. Allosteric ligands can also alter the signalling bias of the orthosteric ligand, even if they have no efficacy on their own. For example, allosteric ligand C has no intrinsic agonism, but when this ligand is cobound with ligand A, a reversal in potency between S1 and S2 is seen (compared to ligand A alone). While ligand A is unable to activate pathway S3, co-binding of both ligands results in activation of this pathway.

significantly potentiates ACh-mediated $_{i}Ca^{2+}$ mobilization and PLC but only weakly potentiates ACh mediated phospholipase D (PLD) responses (Marlo et al. 2009). These examples exemplify the need to understand the effect of allosteric modulation at multiple different signalling pathways, as potentiation/inhibition of all pathways will not necessarily give the desired therapeutic outcome.

For GPCRs that have more than one endogenous ligand and where allosteric ligands display probe dependence, it is possible to observe differential effects on signalling for each allosteric-orthosteric ligand combination. For example, at the GLP-1R, allosteric ligands have the potential to display distinct probe dependent profiles between different endogenous ligands that differs depending on the signalling pathway being assessed (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Knudsen et al. 2007). The ability to modulate all endogenous ligands or to specifically modulate some at the exclusion of others, in a pathway-dependent or independent manner can impact the discovery of allosteric modulators, and can also have implications for the therapeutic application of these ligands.

1.5.3 Challenges of applying allosterism therapeutically

There are without doubt challenges associated with allosteric drug design, in particular with quantification and validation of effects they engender. The majority of compounds are identified through high throughput screens (HTS) of compound libraries, which are extremely costly and time consuming. There is also the need to choose an appropriate assay system to screen biological activity, as allosteric compounds have the potential to engender pathway selectivity, as exemplified by the M_1 mAChR described above (Marlo et al. 2009). This is further illustrated at the CB_1R, where ORG compound positively modulates the binding affinity of the orthosteric agonist CP55940 however, attenuates its efficacy in assays (Price 2005).

The limited structural information available for the family B and C GPCRs impacts the ability to design and predict more refined libraries of compounds that are likely to bind to the receptor of interest. Although there is increasing information of how orthosteric peptide ligands bind at many family B GPCRs, there is still a poor understanding of these receptors on a molecular level, which includes our understanding of mechanistic events associated with receptor activation, location of allosteric binding sites and how binding of ligands to allosteric sites induces modification of receptor function.

It is crucial to investigate probe dependence with all synthetically designed ligands and those ligands found endogenously. Determining the ideal signalling pathways for potentiation at the exclusion of others is of great interest as this provides a novel mechanism to further reduce unwanted side effects profiles, and the desired therapeutic outcome. On the contrary potentiating the wrong pathway may result in higher adverse effects and disease progression. For most receptors the therapeutically relevant pathway(s) is not known and therefore this is one of the key challenges facing drug discovery.

1.6 Pharmacological Quantification of Signalling bias and Allosteric Modulation

1.6.1 Measuring Signalling bias

Activation of GPCRs by ligands does not always result in uniform activation of all signalling pathways mediated by a given receptor (Kenakin & Christopoulos 2013; Christopoulos & Kenakin 2002). It is not surprising that relative to other ligands, many agonists are "biased" towards producing specific subsets of receptor behaviors. Signalling bias is cell type

dependent, thus presenting a particular problem for the characterization of multiple agonists in whole cell test systems removed from the therapeutic one(s). As a consequence of this variability between cell-based responses it becomes difficult to identify and optimize therapeutically meaningful agonist bias. Furthermore, cell-based and tissue based functional selectivity can arise from a single active receptor state due to differences in receptor density. To fully understand signal bias, there is a need for a quantitative scale. One model is provided by application of the Black/Leff operational model (Black & Leff 1983) (Figure 1.7). In this model, response is controlled by two parameters the affinity of the agonist (where K_A is the equilibrium dissociation constant of the agonist-receptor complex) and efficacy (which is the equilibrium constant for the complex formed between the 'activated' receptor and cellular stimulus–response machinery). The efficacy term tau (τ) can be used to describe the intrinsic efficacy of the agonist (i.e. the power of the molecule to induce a response) and the sensitivity of the system to return a response. These two parameters tau and K_A can be combined to give a single number - the transduction ratio log t/K_A as measure of the intrinsic efficacy for an individual pathway (Kenakin 2012; Kenakin et al. 2012). As the activated (agonist-bound) receptor directly interacts with a range of signalling proteins resulting in different cellular signals, each can be quantified by unique τ/K_A ratios that can be used to quantify the bias. Thus, for each pathway these ratios can be calculated and each value can then be normalised to that of a reference ligand. This scale can statistically evaluate selective agonist effects in a manner that can theoretically inform structure-activity studies and/or drug selection matrices (Kenakin et al. 2012).

1.6.2 Measuring allostery

1.6.2.1 The allosteric ternary complex model

As previously mentioned, allosteric ligands display complex behaviours. One of the key challenges associated with the discovery of allosteric ligands is a need to quantify allosteric

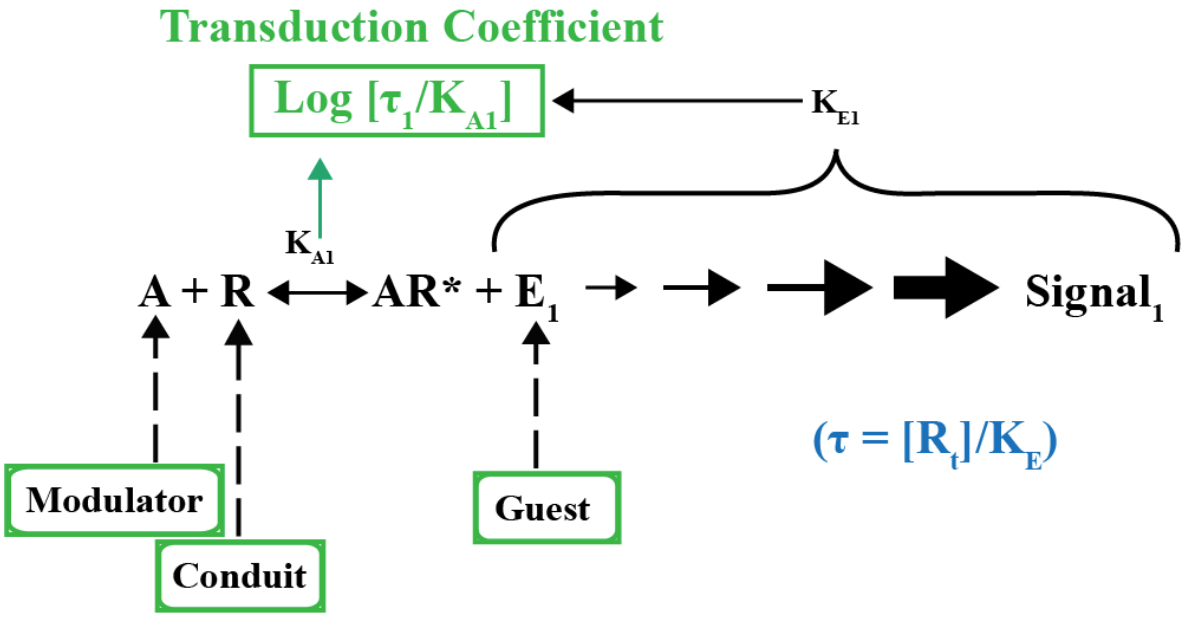


Figure 1.7 A schematic of the Black−Leff operational model to quantify agonist bias

The premise is based on the fact that the receptor conformation stabilized by the agonist will have a unique interaction with all signalling proteins that directly interact with it, thereby setting up the allosteric system(s) of modulator (agonist)/conduit (receptor)/guest (signalling protein). Under these circumstances, the affinity and the efficacy (the "quality" of the conformation) will be determined by the signalling protein, and this will be unique for each pathway. The magnitude of $\log(\tau/K_A)$ will be characteristic of both the affinity and efficacy of the agonist for a particular pathway (Kenakin et al. 2012).

effects. There are a number of pharmacological approaches that can be used either individually or in tandem to detect and successfully quantify allosteric interactions at GPCRs, these include assays to measure binding affinity or to measure different signalling outputs (such as pERK1/2 phosphorylation, cAMP accumulation and Ca^{2+}mobilization) followed by application of analytical methods to assess the results. It is important to measure these effects by using quantitative models to characterise allosteric effects on binding and efficacy in molecular terms, which can then be used to predict allosteric effects in all systems (Kenakin & Christopoulos 2012).

Allosteric models are not as simple as standard orthosteric drug-receptor models in that they must incorporate the potential of the receptor to present different binding sites to different ligands. Many receptor models have been developed to encompass the ability of a protein to adopt multiple conformations that differ in their biological binding/signalling properties, as well as the ability of ligands to selectively enhance distinct subsets of these conformations. The simplest mass-action scheme used to describe an allosteric interaction between two ligands, A and B is the ternary complex model (TCM) (Figure 1.8A). In this model both A and B bind simultaneously to the same receptor (R) at topographically distinct binding sites. Ligand A binds to the orthosteric site with an affinity of K_A, whereas ligand B binds to the allosteric site with an affinity K_B. The magnitude by which the affinity of one ligand is changed by the other ligand when both ligands are co-bound to the receptor, forming the ternary complex (ARB) defines the 'cooperativity factor' symbolized by 'α'. In the TCM model values of $\alpha > 1$ indicate allosteric enhancement of orthosteric ligand affinity (positive cooperativity), whereas values $0 < \alpha < 1$ denote a decrease in affinity (negative cooperativity). Values of $\alpha=1$ indicates no effect on orthosteric affinity (neutral cooperativity) (Christopoulos 2002) . The TCM however is limited in that it only quantifies allosteric effects in terms of binding cooperativity, and not receptor active/inactive states or allosteric effects on orthosteric ligand efficacy. This has lead to the

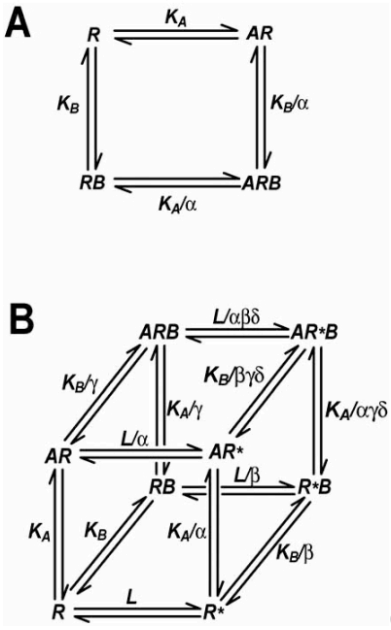
development of the allosteric two-state model (ATSM) (Figure 1.8B).

1.6.2.2 The allosteric two-state model

In addition to exerting effects on affinity, allosteric modulators can modify the signalling efficacy of the orthosteric ligand, thereby extending the parameters of the ATSM to include additional cooperativity factors (L, β, γ, δ, etc). Introducing these factors generates a more complex model with far greater diversity in the repertoire of pharmacological effects that can be achieved by allosteric receptor modulation (Conn et al. 2009). The ATSM encompasses the effects of allosteric ligand affinity, efficacy, and ability to modulate the orthosteric ligand across both receptor states (active/inactive) (Figure 1.8B). 'L' represents the L – isomerization constant describing the transition between *R* (inactive) and *R** (active) receptor states. Symbols 'β' and 'γ' denote cooperativity of efficacy of orthosteric (A) and allosteric (B) ligands to achieve receptor isomerization/activation. 'δ' denotes the activation cooperativity between both ligands A and B to form the active state of the ternary complex. An additional distinguishing feature of the ATSM, is that it can account for allosteric agonism at a receptor unoccupied by an orthosteric ligand (*R*B*). However, this model is limited to conceptualizing allosteric behaviour at receptors as a result of the exhaustive number of parameters and thus, not practical when applied experimentally.

1.6.2.3 The operational model of allosterism

The simplest model for quantifying the minimal number of parameters required to understand allosteric ligand behaviour is an amalgam of the Ehlert model of allosteric receptor effect and the Black–Leff operational model of receptor function (Equation 1) (Black & Leff 1983; Leach et al. 2007). In this model A, B, K_A, K_B, and α remain the same as in the ATCM, however, additional parameters include *E*, E_m, β, τ_A, and τ_B. The parameter *'E'* represents the effect and *'E_m'* represents the maximal response of the system. The operational model further extends the



(Gregory et al. 2007)

Figure 1.8 Models of allosteric interaction

The major schemes describing allosteric interactions. **A,** the allosteric ternary complex model (ATCM), **B,** the allosteric two-state model (ATSM). In both models, *R* denotes inactive receptor conformations, *R** denotes active receptor conformations, and *L* is the isomerization constant describing the transition between *R and R** states. *A* and *B* represent orthosteric and allosteric ligands, for which their binding affinities are denoted by K_A and K_B. α describes the cooperativity in binding between orthosteric and allosteric ligands, respectively. β and γ describes the cooperativity in efficacy of the orthosteric and allosteric ligand to achieve receptor isomerisation/activation, respectively. δ denotes the activation cooperativity between both ligands to form the active state of the ternary complex (Keov et al. 2011; Hall 2000).

ATCM to include 'S', which represents the stimulus of the system, and the cooperativity parameter *'β',* which reflects the modulation between ligands in efficacy. *β* describes the magnitude by which the allosteric modulator modifies the efficacy of the orthosteric ligand in the ARB ternary complex and must account for system variables such as receptor concentration and ability of the orthosteric and allosteric ligands to stimulate the system(s) (extent of agonism). These system variables are described by two 'transducer' parameters 'τ_A' and 'τ_B' for orthosteric and allosteric ligands respectively. Low τ_B values indicate low receptor expression and/or low coupling efficacy in the system, whereas high τ_B values indicate high receptor expression and/or high coupling efficacy; changes will be observed in basal response, reflective of allosteric agonism, and orthosteric ligand potency, but possibly not in maximal response. In both cases allosteric effects may not be evident, as the system may have already achieved its maximal stimulation (E_m) in the presence of the orthosteric agonists (Keov et al. 2011).

Equation 1.

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

This model can be applied to functional data where orthosteric ligand concentration response curves are performed in the presence of increasing concentrations of allosteric ligand. From these data, K_A, K_B, τ_A, τ_B, α and β can be calculated, however, a direct estimation of the cooperative effect on affinity can also be derived via equilibrium and dissociation binding experiments (Kenakin & Christopoulos 2012).

SECTION 2:

1.7 Glucose homeostasis and the incretin system

1.7.1 General introduction

Glucose is the body's primary source from intestinal absorption and can be metabolized directly from the digestive system during the fed state (glycolysis), or can be produced by the body during nutrient absence (gluconeogenesis) (reviewed by (Baggio & Drucker 2007; Reimann 2010)). The pancreas plays a crucial role in regulating each of these processes, acting as both an endocrine gland and a digestive organ and is responsible for producing multiple important hormones involved in glucose homeostasis. This dual-functional gland is made up of millions of cell clusters called islets of Langerhans that contain α-cells that secrete glucagon and β-cells that secrete insulin. Glucose homeostasis is governed by the interplay of insulin and glucagon and also involves other hormones such as amylin and other incretin hormones (e.g. GIP). Glucagon promotes the release of glucose into the plasma and its secretion is stimulated and inhibited in response to low (hypoglycemia) and high (hyperglycemia) circulating levels of glucose in the blood respectively (Brubaker & Drucker 2002).

Insulin is a small anabolic hormone, secreted in response to increased blood glucose and amino acids following an ingested meal. Insulin exerts its actions through binding to a specific receptor on many cells through the body, in particular adipose tissue, liver and muscle (Aronoff et al. 2004) (Baggio & Drucker 2007). Pancreatic β-cells respond instantly to increases in plasma glucose concentrations, by releasing corresponding amounts of insulin, thereby regulating metabolic processes, such as peripheral glucose uptake, lipid synthesis, or inhibition of hepatic gluconeogenesis period. Insulin maintains glucose homeostasis by three highly synchronized processes, (i) enhancing glucose uptake by the liver and gut (ii) augmenting the uptake of

glucose by the peripheral (muscle) tissues and (iii) suppression of hepatic glucose production (DeFronzo 1992). The entero-insular axis describes the interplay between the gastrointestinal tract (GIT) and the pancreas and is crucial to these mechanisms to occur (Figure 1.9).

1.7.2 The entero-insular axis and the incretin effect

The entero-insular axis describes communication specifically between the GIT and pancreatic β-cells, a relationship that is mediated by incretin hormones (Unger & Eisentraut 1969). The 'incretin effect' refers to incretin hormones being secreted from the GIT into the circulation in response to nutrient ingestion, thereby enhancing glucose-stimulated insulin secretion. This phenomenon is highlighted by the fact that oral nutrient (glucose) administration promotes a much larger degree of insulin secretion in comparison to intravenous glucose infusion. The incretin effect accounts for ~50-70% of the total insulin secreted following oral glucose administration. Studies have recognised that while arterial glucose concentration stimulated insulin secretion, incretins released from the gut in response to glucose absorption sensitised pancreatic β-cells, subsequently reducing the threshold for release of insulin (Ranganath 2008).

1.7.3 Incretin hormones

Incretins are hormones primarily released from the GIT into the bloodstream in response to nutrient intake. From there they act with products of food ingestion to modulate insulin secretory responses such as promoting cell signalling, leading to insulin biosynthesis and subsequent secretion from pancreatic β-cells. The principal incretin hormones include glucose-dependent insulinotropic peptide (GIP) and GLP-1.

GIP is a 42-amino acid peptide derived from the post-translational processing of a 153-amino acid precursor encoded by the *gip* gene (W. Kim & Egan 2008; Takeda et al. 1987). This was the first incretin to be isolated from intestinal mucosa and its insulinotropic properties characterised

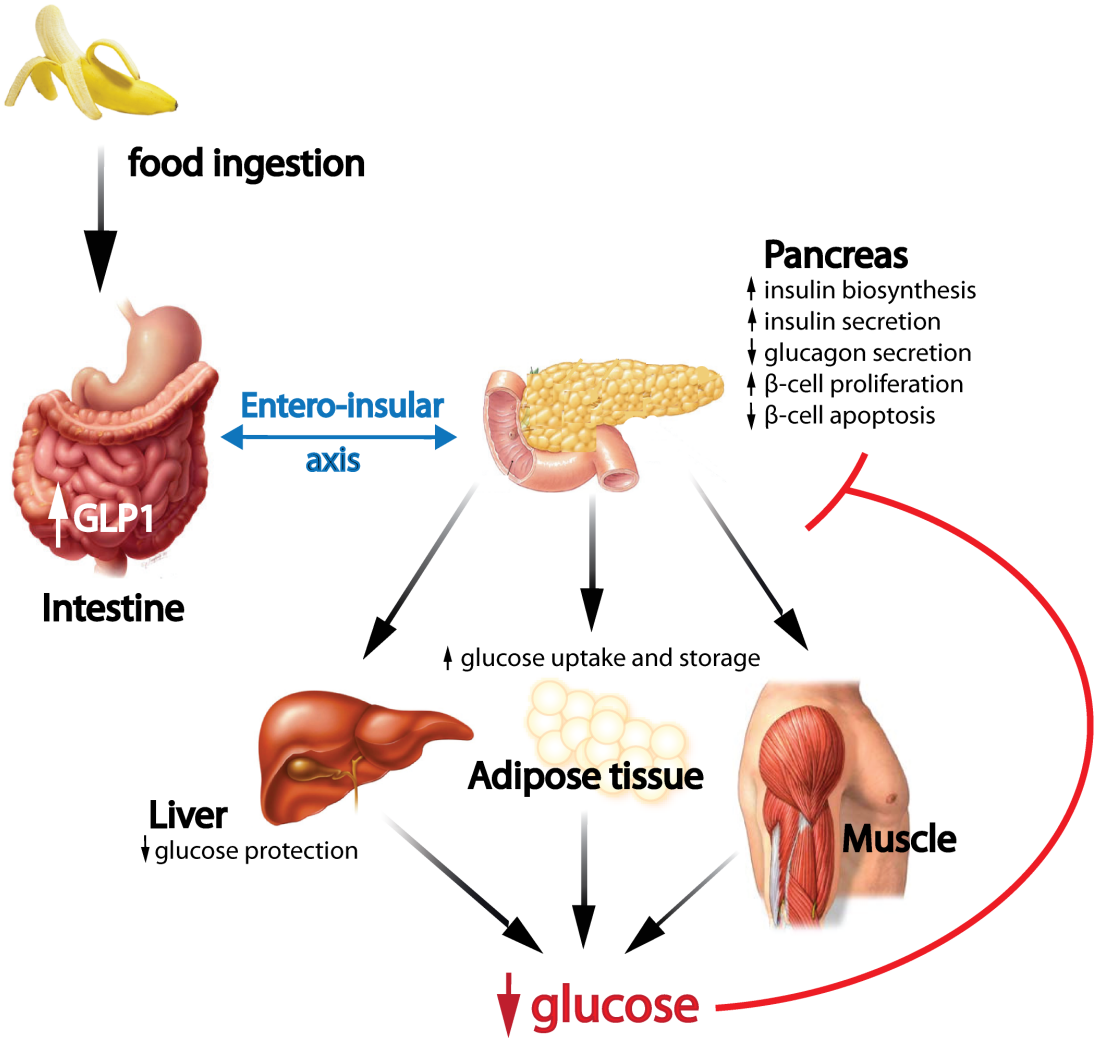


Figure 1.9 Control of human plasma glucose levels via the entero-insulin axis.

Ingestion of a glucose-rich meal augments secretion of incretins from the GIT. Incretins, such as GLP-1, subsequently perform glucoregulatory activities via the pancreas, liver, adipose tissue and muscle. This results in a decrease in plasma glucose that acts in a negative feedback manner to inhibit further insulin release from the pancreas.

(Brown & Dryburgh 1971; W. Kim & Egan 2008). GIP is synthesised and released in response to nutrients from the enteroendocrine cells (called K cells) primarily in the proximal small intestine (duodenum and jejunum). This peptide inhibits gastric acid secretion and possesses glucose-dependent stimulatory effects on insulin secretion (Brown & Dryburgh 1971; W. Kim & Egan 2008).

Following the discovery of GIP, GLP-1 was identified during the cloning and characterisation of the proglucagon gene (Bell, Santerre, et al. 1983b; Bell, Sanchez-Pescador, et al. 1983a). Like GIP, GLP-1 potentiates glucose-mediated insulin release in an additive manner and together these hormones explain the incretin effect observed in humans (Nauck et al. 1993; Vilsbøll et al. 2003). GLP-1 is secreted from intestinal endocrine L-cells, which are located mainly in the distal ileum and colon. Secretion is stimulated by a variety of nutrient, neural, and endocrine factors. Release of GLP-1 can be stimulated by mixed meals or individual nutrients including glucose and other sugars, fatty acids, essential amino acids, and dietary fiber consumed orally (Baggio & Drucker 2007).

Even though GIP and GLP-1 are the only two classified incretins, many other proglucagon derived peptides such as glicentin, glucagon, oxyntomodulin, and GLP-2 represent physiologically important regulators of nutrient intake and digestion and are currently being used clinically or under active investigation. GLP-2 is related to GLP-1 and is co-secreted from gut L cells primarily in response to nutrients. GLP-2 regulates gastric motility, gastric acid secretion, intestinal hexose transport, and increases the barrier function of the gut epithelium. Initially GLP-2 was classified as an incretin, however clinical and experimental observations revealed no influence on insulin expression/biosynthesis or secretion (Drucker et al. 1996; Drucker 2001).

1.8 The glucagon-like peptide-1 (GLP-1) system

1.8.1 Physiological functions of GLP-1

GLP-1 exerts its effects via binding to the glucagon like peptide-1 receptor (GLP-1R). This interaction results in amplification of intracellular signalling components that ultimately leads to increased expression, biosynthesis and secretion of insulin from pancreatic β-cells in a glucose-dependent manner. In addition to its insulinotropic effects, GLP-1 inhibits gastric emptying, subsequently inducing satiety and reducing food intake (Willms et al. 1996). Furthermore, GLP-1 inhibits glucagon secretion (Komatsu et al. 1989), thus slowing the rate of endogenous glucose production (Prigeon et al. 2003) both of which result in lowered blood glucose. GLP-1 also acts to protect β-cells from apoptosis (Farilla 2002) and stimulates β-cell proliferation. One way by which GLP-1R can stimulate β-cell proliferation is through regulation of the β-cell transcription factor pancreatic duodenal homeobox-1 (PDX-1) (Perfetti et al. 2000; Stoffers et al. 2000), which also augments insulin gene transcription and up-regulation of glucokinase (GK) and glucose transporter2 (GLUT2) (X. Wang et al. 1999; W. Kim & Egan 2008). This has been confirmed in GLP-1R knockout mice (GLP-1R$^{-/-}$) that display normal β-cell mass, however altered islet cell topography, defective β-cell regeneration, reduced glucose tolerance and increases in glucagon producing α-cells (Y. Li et al. 2003; Ling et al. 2001). This highlights the importance of the GLP-1R and GLP-1 in islet cell maintenance and development. Consistent with this, continuous GLP-1 administration into diabetic rodent models shows an increase in islet size and β-cell mass, and a substantial decrease in apoptotic β-cells (Farilla 2002), while also enhancing glucose sensitivity in β-cells (Holz et al. 1993).

In addition to pancreatic effects, GLP-1 also exerts biological actions in both the central and peripheral nervous systems, the gastrointestinal system, cardiovascular system, muscle, adipose tissue and liver, brain (hypothalamus), respiratory system, pulmonary arteries and kidney, each

of which express the GLP-1R (Alvarez et al. 2005; Dunphy et al. 1998; Y. Wei & Mojsov 1995) (Figure 1.10). In the nervous system, GLP-1 has a role in control of ingestive behavior and appetite regulation (Larsen et al. 1997). In addition, GLP-1 augments neogenesis, proliferation and anti-apoptotic behavior of neuronal cells and subsequently enhances memory, spatial and associative learning in rodents (During et al. 2003; T. Perry 2002). In addition, GLP-1R agonists can preserve primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinson's (Harkavyi et al. 2008; Bertilsson et al. 2008; Y. Li et al. 2009). The GLP-1 mimetic, exendin-4, decreases brain damage and provides neuronal protection from metabolic and oxidative insults, improving functional outcomes, in a transient middle cerebral artery occlusion stroke mouse model (Rampersaud et al. 2012; Aviles-Olmos et al. 2013; Y. Li et al. 2012b).

In the GI system, GLP-1 inhibits gastric emptying. This is particularly important as it allows regulation of nutrient content in small intestine and consequently levels of GLP-1 and insulin secretion (Wettergren et al. 1993). This is also mediated by the nervous system; sensory information in the GIT is relayed to the brain and hypothalamus where hormones are released to assist in the management of gastric emptying (Larsen et al. 1997).

In muscles, adipose and hepatic cells, GLP-1 augments glycogen synthase activity and therefore favors conversion of glucose into glycogen (Baggio & Drucker 2007). GLP-1 and GLP-1 related agonists also enhance peripheral insulin sensitivity and reduce steatosis (Young et al. 1999). In addition, GLP-1 functions to decrease hepatic glucose production, which in combination with the effects observed in muscle and adipose tissue, help in plasma glucose reduction (Prigeon et al. 2003).

In the cardiovascular system, central and/or peripherally administrated GLP-1 and related

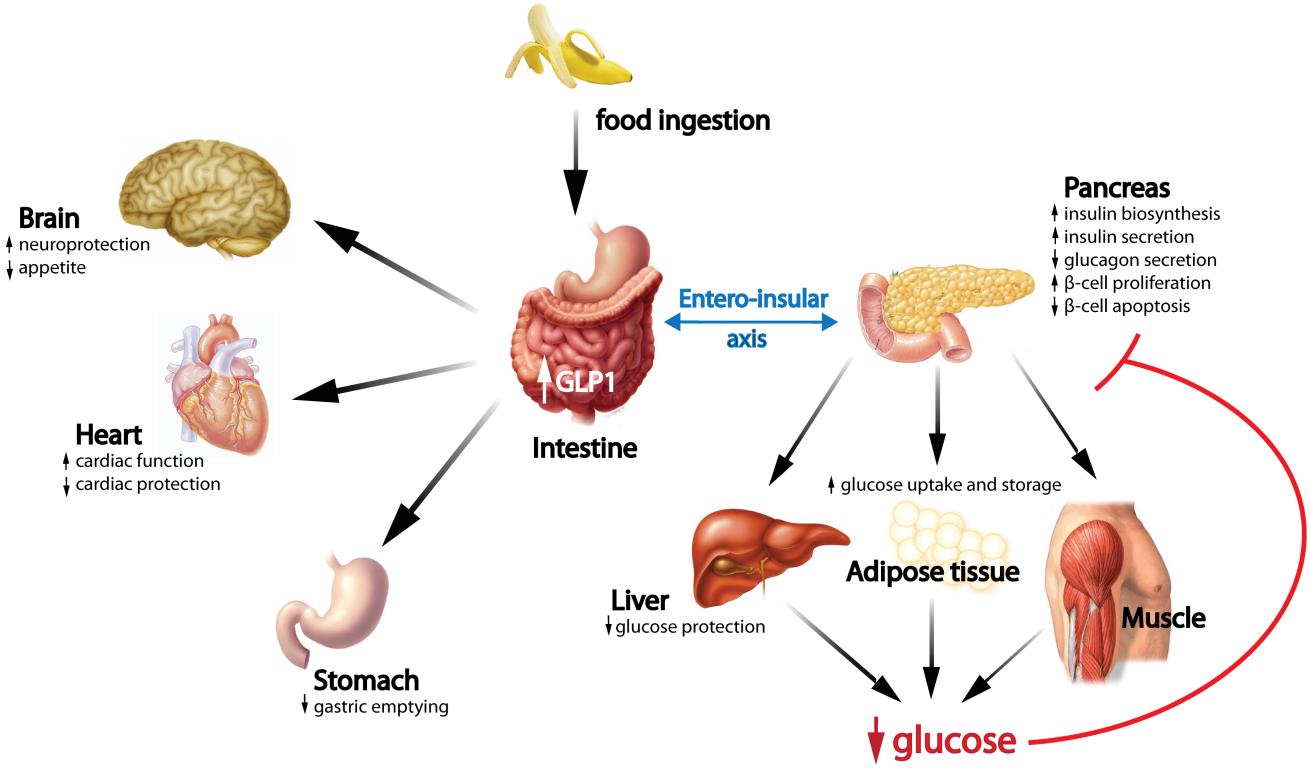


Figure 1.10 Peripheral actions of GLP-1

GLP-1 is not only involved in regulation of glucose but also has additional actions throughout the body. In this figure, GLP-1 can act in the brain causing neuroprotection and appetite suppression, while in the heart GLP-1 acts to increase cardiac function, thus providing cardiac protection. Furthermore, GLP-1 acts at the stomach (both directly and via the CNS) to reduce gastric emptying.

analogues increase blood pressure and heart rate, mediated through neuroendocrine and autonomic control via vagal nerves (Barragán et al. 1994; Barragán et al. 1999; Yamamoto et al. 2002). In addition to improving cardiac contractility, GLP-1 attenuates endothelial dysfunction in both human and animal models (Barragan *et al.*, 1994; Bose *et al.*, 2005; Nikolaidis *et al.*, 2005a; Nikolaidis *et al.*, 2005b; Sokos *et al.*, 2006). GLP-1R$^{-/-}$ mice exhibit reduced resting heart rate, elevated left ventricular (LV) end diastolic pressure, increased LV thickness and impaired contractile responses to insulin and epinephrine (Ban *et al.*, 2008). GLP-1 increases myocardial insulin sensitivity (Nikolaidis *et al.*, 2004), including myocardial glucose uptake independently of plasma insulin levels (Bhashyam *et al.*, 2010). The survival of cardiac myocytes induced by GLP-1 agonists is mediated by inhibition of apoptosis via cAMP and PI3-K pathways (including activation of Akt), following the binding to GLP-1Rs (Bose *et al.*, 2005). Activation of the Akt mediator (serine-threonine kinase) attenuates cardiomyocyte death, restores regional wall thickening after myocardial ischaemia and improves survival of preserved cardiomyocytes (Matsui *et al.*, 2001).

In the respiratory system, GLP-1 induces pulmonary relaxation and is involved in mucus production in the lungs and trachea (Richter et al. 1993), while in the kidney, GLP-1 increases the rate of glomerular filtration/sodium excretion, thereby inhibiting the development of hypertension and improving endothelial integrity (Gutzwiller et al. 2004; Gutzwiller et al. 2006).

1.8.2 The GLP-1 receptor (GLP-1R)

The GLP-1R is a 463-amino-acid transmembrane-spanning protein belonging to the family B/secretin GPCRs. There are currently no full length family B GPCR crystal structures solved, however for the majority of secretin-like family B GPCRs, there are structures of the N-terminal domain that forms the orthosteric binding site. For the GLP-1R, these isolated GLP-1R N-

terminal structures are in complex with orthosteric α-helical peptides GLP-1 or exendin-4, confirming the importance of the N-terminal domain in peptide binding (Runge et al. 2008; Underwood et al. 2010). In these structures, the C-terminus of the peptide interacts with the receptor N-terminal domain, while the N-terminus of the peptide is thought to associate with the core of the receptor, predominantly the ECLs to influence transmission of signal and signalling specificity to intracellular effectors (Al-Sabah & Donnelly 2003; Coopman et al. 2011). This broadly recognized two-domain model of ligand binding is also supported experimentally by chimeric receptors (Runge et al. 2003), photolabile peptide cross-linking (Q. Chen et al. 2009; Q. Chen et al. 2010; L. J. Miller et al. 2011), and by mutagenesis analysis (López de Maturana & Donnelly 2002; Koole, Wootten, Simms, Savage, et al. 2012b; Koole, Wootten, Simms, Miller, et al. 2012a). Although there are no TM bundle structures of the GLP-1R, the recent structures of the CRF1R and GCGR TM domains allows for homology modeling of the TM bundle of the closely related GLP-1R that may allow for a better mechanistic understanding of receptor function (Siu et al. 2013; Hollenstein et al. 2013).

1.8.3 GLP-1R signalling and regulation

The GLP-1R is primarily expressed on the surface of pancreatic β-cells, where it is responsible for regulating glucose-dependent insulin biosynthesis and release, inducing β-cell proliferation and neogenesis, decreasing β-cell apoptosis and inhibiting glucagon secretion. The GLP-1R is pleiotropically coupled to multiple G proteins, including Gαs, and to a lesser extent Gαq and Gαi proteins (Montrose-Rafizadeh et al. 1999). Activated G proteins promote the generation of second messengers such as cyclic adenosine monophosphate (cAMP), Ca^{2+}, or phosphoinositides, in addition to the phosphorylation of MAP kinase pathways (including ERK1/2 phosphorylation) downstream of GLP-1R activation (Neer 1995).

The GLP-1R is primarily coupled to $G\alpha_s$ heterotrimeric G-proteins, resulting in activation of AC and generation of cAMP. This subsequently promotes activation of protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor II (Epac2) that are directly involved in enhancing proinsulin gene transcription and subsequent insulin biosynthesis/secretion (Doyle & Egan 2007; Holst 2007; Holz 2004; Kashima et al. 2001; Seino 2005; Fehmann & Habener 1992). In addition, activated PKA phosphorylates and directly inhibits K_{ATP} channels, which is further assisted by Epac2/Rap1/PLCε (Light et al. 2002; Dzhura et al. 2010; Leech et al. 2011). Closure of the K_{ATP} channels increases intracellular Ca^{2+} as result of Ca^{2+} influx through voltage gated Ca^{2+} channels (VGCC), cation channels and mobilization of intracellular Ca^{2+} stores. These combined effects further increase cytosolic Ca^{2+} and subsequent membrane depolarization of β-cells results in exocytosis of insulin. Continuing GLP-1-mediated increases in production of mitochondrial ATP negatively regulates voltage-dependent K^+ (Kv) channels and Kv currents preventing β-cell repolarization, which has direct effects on insulin storage in β-cells. GLP-1 also promotes Ca^{2+}-induced Ca^{2+} release through IP_3 receptors in response to Gq and Gs protein activation via downstream activation of PKA and PLC mediated phosphorylation events (G. Kang et al. 2003; Tsuboi et al. 2003; Koole et al. 2012). Unpublished inhibitor data from out lab displays the role of Gs and Gi in cAMP accumulation, Gq, Gi, Gβγ and Gs in Ca^{2+}, while Gq, Gi, Gβγ and β-arrestin1 and βarrestin2 are involved in ERK1/2 phosphorylation. CREB activation (downstream of cAMP) of Bcl-2 and Bcl-xl are also known to be involved in β-cell proliferation, while neogenesis is primarily linked with PKA activation of MAPK and cyclin D1 (an important regulator of cell cycle progression from G1 to S-phase). Inhibition of β-cell apoptosis has been reported to be regulated by Akt/PKB though inhibition of caspase activation, as well as inhibiting activation of NFkB and Foxo1. ER stress reduction is associated with ATF-4 activation by PKA, which then stimulates CHOP (C/EBP homologous protein) and Gadd34, preventing dephosphorylation of pelF2α (Baggio & Drucker 2007; Portha et al. 2011). GLP-1

also exerts its stimulatory effects on β-cell proliferation through CREB-mediated IRS2 gene expression, leading to activation of PI-3K/PKB (Portha et al. 2011). PI3K and subsequent ERK1/2 activity have been linked to mediating β-cell proliferation/differentiation (Arnette 2003; Buteau et al. 2001; Gomez 2002; Klinger et al. 2008; Park et al. 2006; Quoyer et al. 2010). Other outcomes of GLP-1R activity are promotion of insulin gene transcription, nuclear translocation of PKCζ and EGF receptor transactivation (Khoo 2003; Baggio & Drucker 2007; Buteau et al. 2003) (Figure 1.11).

Emerging evidence also reveals a role for β-arrestins and GRKs in GLP-1 mediated biological effects (Feng et al. 2011). In particular, β–arrestin1 knockdown using siRNA in pancreatic β-cells results in a decrease in ERK/CREB activation, IRS-2 expression, cAMP production and insulin secretion following GLP-1R activation. A separate study revealed that GLP-1R activation induces two distinct ERK1/2 phosphorylation responses. The first, a PKA-dependent pathway that mediates rapid and transient ERK1/2 phosphorylation leading to nuclear translocation of the activated kinase and the second, a late ERK1/2 response mediated by β-arrestin 1 that is restricted to the β-cell cytoplasm. Furthermore, this study found that β-arrestin 1-dependent ERK1/2 signalling engaged by GLP-1 stimulates p90RSK activity, mediating the phosphorylation of *Bcl-2*-associated death promoter (Bad) that has an anti-apoptotic role (Quoyer et al. 2010).

There is limited evidence that GLP-1R internalisation/desensitisation is independent of β-arrestin mediated events (Sonoda et al. 2008), but instead GLP-1R endocytosis is dependent on caveolin-1 (Syme et al. 2006). The role that β-arrestins and GRKs play in this process (if any) is still unclear. After internalisation, GPCRs are either desensitised and recycled back to the membrane or they are down regulated by lysosomal/proteosomal degradation. For the GLP-1R, these processes are poorly understood. Furthermore, little is known on the roles of GRKs and β-

arrestins in downstream signal transduction and all prior studies on regulation and arrestin recruitment/signalling have predominantly only focused on using GLP-1(7-36)NH_2 as an agonist, despite there being multiple endogenous ligands (at least six) and exogenous ligands that are used clinically for this receptor.

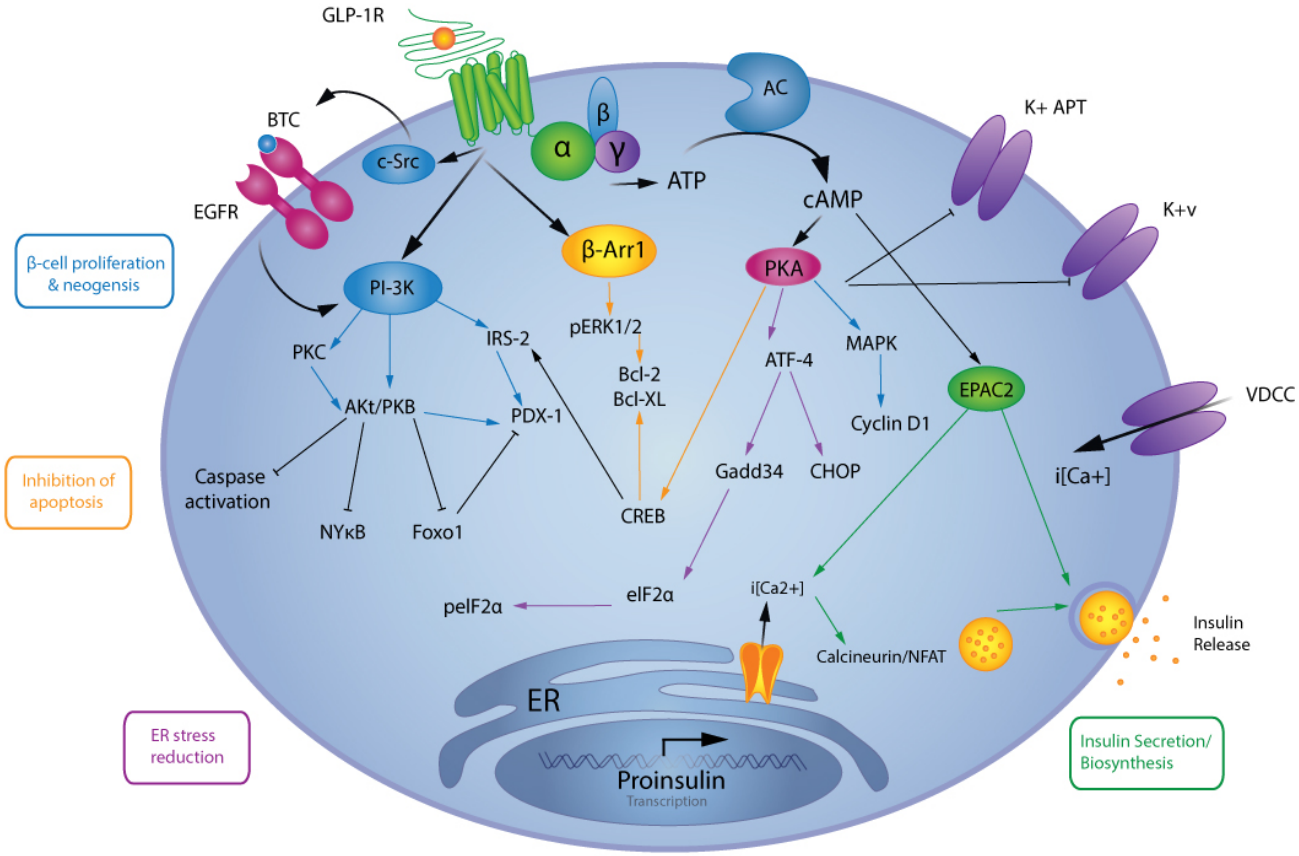


Figure 1.11 Pancreatic β–cell signalling pathways

The pancreas is a therapeutic target for type 2 diabetes, as it has multiple signalling pathways that lead to beneficial physiological outcomes**.** GLP-1R activation of these pathways may lead to β-cell proliferation and neogenesis (blue), inhibition of apoptosis (yellow), ER stress reduction (pink) and insulin secretion/biosynthesis (green).

1.9 GLP-1R Ligands

1.9.1 GLP-1 peptides

There are multiple endogenous ligands that target and activate the GLP-1R, all of which are highly conserved between species (identical across mice, rats and humans) (Drucker et al. 1987). Post-translational processing of the glucagon precursor (proglucagon) within the intestinal L-cells gives rise to GLP-1(7-36)NH_2 and its related non-amidated glycine-extended peptide GLP-1(7-37). These two forms of GLP-1 are secreted from the gastrointestinal tract and account for the majority of secreted GLP-1 (approximately 80%), are equipotent and are the primary insulinotropic peptides, stimulating insulin and inhibiting glucagon secretion, effects which are glucose dependent (Orskov *et al.*, 1993; (Deacon, Nauck, et al. 1995b)Kieffer *et al.*, 1999; Koole *et al.*, 2011; Orskov *et al.*, 1993). Other variants of GLP-1 include full length N-terminal extended GLP-1(1–37) and GLP-1(1-36)NH_2, both of which are primarily secreted from the pancreas, possess lower affinity for the GLP-1R than the truncated forms and exert little/no effect on insulin secretion (Orskov et al. 1994). Although their function remains unclear, studies have shown GLP-1(1-37) converts intestinal epithelial cells into insulin-producing cells (Suzuki *et al.*, 2003). Radio-immunoassays revealed that, of the two bioactive insulin-releasing peptides, 80% corresponded to GLP-1(7-36)NH_2 and 20% to GLP-1(7-37) (Orskov et al. 1994; ØRSKOV et al. 1986). While GLP-1(7-36)NH_2 and GLP-1(7-37) posses glucose-dependent insulinotropic effects, GLP-1(7-37) has a half-life 2/3 that of GLP-1(7-36)NH_2, suggesting that amidation is an important mechanism to enhance peptide survival in the plasma (Wettergren et al. 1998). Plasma levels of GLP-1(7-36)NH_2 and to a lesser extent GLP-1(7-37) range from 5-10 pmol/L increasing to 15-30 pmol/L post feed.

In addition to the different variants of GLP-1 peptides, other endogenous peptides such as glucagon, GIP and oxyntomodulin also bind to the GLP-1R. Glucagon and GIP both posses very

low affinity, (glucagon affinity is 1000-fold less than that of GLP-1) (Thorens 1992; W. Kim & Egan 2008). Oxyntomodulin also activates the GLP-1R with a lower affinity than truncated GLP-1 peptides. Furthermore, this peptide also has weak affinity for the glucagon receptor, however the GLP-1R is thought to be the primary receptor for oxyntomodulin activity and this peptide has a high degree of sequence homology with GLP-1 (Figure 1.12) (Baggio et al. 2004).

1.9.2 Degradation and clearance of GLP-1 and analogues

In vivo, GLP-1 is extremely susceptible to rapid degradation and clearance with a half-life between 2-6 minutes (Orskov et al. 1993). This is not only a result of catalytic activity of the enzyme serine protease dipeptidyl peptidase IV (DPPIV) but also neutral endopeptidase 24.11(NEP), glomerular filtration via the renal system and hepatic clearance (Hupe-Sodmann et al. 1995; Deacon, Johnsen, et al. 1995a; Kieffer & Habener 1999). In particular, DPPIV cleaves the two NH_2-terminal amino acids from all forms of GLP-1 to form GLP-1(9–36)NH_2 and GLP-1(9-37). These forms of GLP-1 have very low affinity for the GLP-1R (~1000 fold lower than GLP-1(7-36)NH_2 and GLP-1(7-37)) and do not promote insulin release (Wettergren et al, 1998). Due to the activity of DPPIV, only 10-15 % of secreted GLP-1 reaches systemic circulation (Deacon, Johnsen, et al. 1995a; Kieffer et al. 1995; Mentlein et al. 1993). Similar to GLP-1, oxyntomodulin is targeted for degradation by DPPIV and NEP (Hupe-Sodmann et al. 1995; Zhu et al. 2003).

1.9.3 Exogenous orthosteric peptide ligands of the GLP-1R

Exendin-4 is a naturally occurring GLP-1R agonist extracted from the salivary glands of the lizard, *Heloderma suspectum* (Gila monster) (Eng et al. 1992). Exendin-4 shares 53% sequence homology with GLP-1 (Figure 1.12) and is a powerful stimulant of glucose-dependent insulin secretion (Göke et al. 1993; W. Kim & Egan 2008). Exendin-4 shares comparable physiological

Figure 1.12 Amino acid sequence of the major GLP-1 peptides

The four endogenous forms of GLP-1, two of which are glycine extended at the C-terminus (GLP-1(1-37) and GLP-1(7-37)), and two of which are C-terminally amidated (NH_2) (GLP-1(1-36)NH_2 and GLP-1(7-36)NH_2). DPPIV degradation of GLP-1 yields N-terminally truncated metabolites GLP-1(9-37) and GLP-1(9-36)NH_2. The endogenous agonist oxyntomodulin and exogenous agonist exendin-4 share high homology in the N-terminal region of the peptide, highlighted in red.

properties to GLP-1, including promotion of β-cell proliferation and augmentation of insulin synthesis/secretion (Drucker 2003b). One important distinction between these peptide sequences is that exendin-4 possesses a glycine at residue two, corresponding to an alanine in GLP-1. This subtle difference in sequence makes exendin-4 resistant to the proteolytic activity of DPPIV, thereby resulting in increased half-life *in vivo* (Drucker 2003a). N-terminally truncated exendin-4 yields an antagonist of the GLP-1 receptor (exendin (9-39)) (Göke et al. 1993). Exendin(9-39) causes hyperglycemia in animal and humans during fasting as a consequence of antagonism of GLP-1-mediated insulin synthesis and secretion (Baggio et al. 2000).

1.9.4 Synthetically designed peptides

Due to its broad profile of beneficial physiological effects, the GLP-1R has attracted substantial attention as a target for T2DM, (refer to later section of diabetes mellitus). Development of synthetic peptides with specificity for the GLP-1R with enhanced stability and/or function has been a goal in pharmacological research. Currently there are GLP-1R agonists and GLP-1 analogs at various stages of preclinical or clinical development that are resistant to DPPIV degradation and NEP hydrolysis. There have been multiple investigations around modifying GLP-1 at positions His^{7}, Ala^{8}, or Glu^{9}, as DPPIV cleaves peptides at alanine, proline, or hydroxyproline in the penultimate N-terminal positions. Examples of these GLP-1R agonists/GLP-1 analogues that display insulinotropic activity and enhanced metabolic stability include (Val^{8})GLP-1, (Thr^{8})GLP-1, (Ser^{8})GLP-1, (Gly^{8})GLP-1 and (Ser^{2}) oxyntomodulin (Green et al. 2004).

Additional peptide modifications through fatty acid derivatisation result in extended biological activity in the plasma. liraglutide, a long-acting GLP-1 analog with 97% homology to native GLP-1, has a substitution of Lys^{34} with Arg^{34} that allows the attachment of a C-16 free-fatty acid derivative via a glutamoyl spacer to Lys^{26}. The free-fatty acid derivative promotes non-covalent

binding of Liraglutide to albumin, subsequently increasing plasma half-life to up to 15 h (W. Kim & Egan 2008). Liraglutide significantly improves glycemic control and promotes weight loss in humans/mice/rats, as well as enhancing β-cell function. Other GLP-1 analogs that with insulinotropic activity and enhanced metabolic stability include: LY315902, LY2189265, CJC-1131 and albumin-conjugated dimeric GLP-1 albiglutide (Seino et al. 2009; Barrington, Chien, Tibaldi, et al. 2011b; Barrington, Chien, Showalter, et al. 2011a; Chou et al. 1997; J.-G. Kim et al. 2003; Matthews et al. 2008; Näslund et al. 2002; Rosenstock et al. 2009). Analogues of oxyntomodulin that have improved stability have also been developed and include: AC3174, CJC-1134-PC and OXM6421 (Baggio et al. 2008; Christensen & Knop 2010; Hargrove et al. 2007; Kerr et al. 2010; Y.-L. Liu et al. 2010). In addition, a series of small 11mer peptides developed by BMS acutely reduce plasma glucose excursions and increase plasma insulin concentrations in mouse models of diabetes (Gigoux & Fourmy 2013; Mapelli et al. 2009). These peptides also showed enhanced pharmacokinetic half-life relative to GLP-1, over several hours in mouse and dog models (Mapelli et al. 2009).

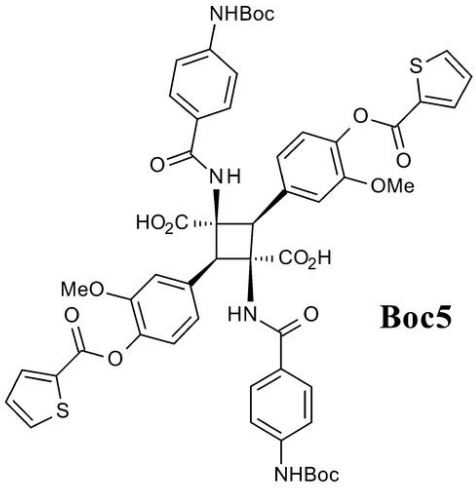
Therapeutic development of peptides remains a significant challenge in drug development, as peptide stability and route of administration are difficult and complex. Furthermore, GLP-1 peptide analogues are coupled to some extent with undesirable and detrimental side effects, the most prominent being nausea and pancreatitis (Aroda & Ratner 2011).

1.9.5 Non-peptide and allosteric ligands

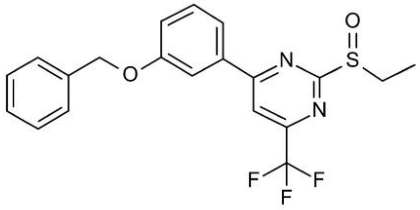
All of the previously described GLP-1R ligands are peptides. There is increasing emphasis on development of non-peptide ligands that can be administered orally. Functional screens have identified GLP-1R small molecule agonists. These include cyclobutanes, Boc5 and S4P (Figure 1.12) (D. Chen et al. 2007). Boc5 is fully efficacious across a range of physiological endpoints but has lower affinity and potency than native GLP-1. Boc-5 decreases plasma glucose, controls

nutrient intake and subsequently aids in weight loss in obese mice (Su, He, Li, Liu, J. Wang, Y. Wang, W. Gao, Zhou, Liao, Young & M.-W. Wang 2008b; He et al. 2012; Ge et al. 2013). Evidence suggests these compounds bind to the N-terminal domain of the receptor but it is unclear whether these compounds bind to the orthosteric or an allosteric binding site, although there is limited evidence to suggest a potential allosteric mode of binding due to the inability these compounds to fully inhibit ^{125}I-GLP-1(7-36)NH_2 binding (D. Chen et al. 2007). Compound 1 (2-(2'-methyl)thiadiazolylsulfanyl-3-3trifluoromethyl-6,7-dichloroquimozaline) and Compound 2 (6,7'-dichloro2-methylsulfonyl-3-*tert*-butylaminoquinoxaline) (Figure 1.13) were identified by Novo Nordisk as two non-peptide allosteric agonists of the GLP-1R that demonstrated glucose-dependent insulin release *in vivo* (Knudsen et al. 2007). (4-(3-(benzyloxy)phenyl)-2-(ethylsulfinyl)-6-(trifluoromethyl)pyrimidine), BETP (Figure 1.13) identified by Eli Lilly also has an allosteric mode of binding and increases glucose-dependent insulin release from normal and diabetic human islets (Sloop et al. 2010). A recent study has reported Compound 2 and BETP to interact with the intracellular face of the GLP-1R, where they covalently modify cysteine residues C347 and C438 in the ICL3 (Nolte et al. 2014).

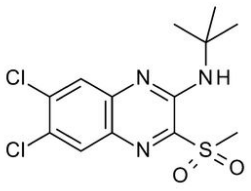
Additional small molecule non-peptide ligands have also been identified and include a series of compounds developed by TransTech Pharma, the most promising of which is TTP054, (structurally similar to T-0632) (Figure 1.12), which had robust effects on glycemic control in multiple studies in type 2 diabetics not well controlled on metformin, and is currently being evaluated in a dose ranging study intended to inform on the design of phase 2b POC (proof of concept) studies (TransTech Pharma, Inc). T-0632 is an allosteric inhibitor expressing no intrinsic activity of its own, yet acting as a non-competitive inhibitor of GLP-1 induced cAMP response (Tibaduiza et al. 2001). Despite this, T-0632 (Figure 1.13) lacks receptor selectivity as it displays antagonistic properties at the cholecystokinin CCK-1 receptor (Taniguchi et al. 1997).



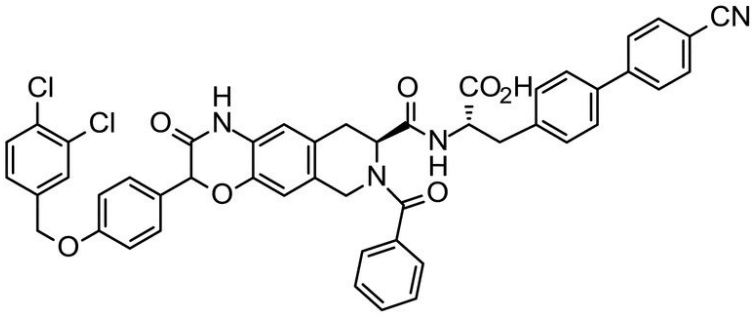
Boc5



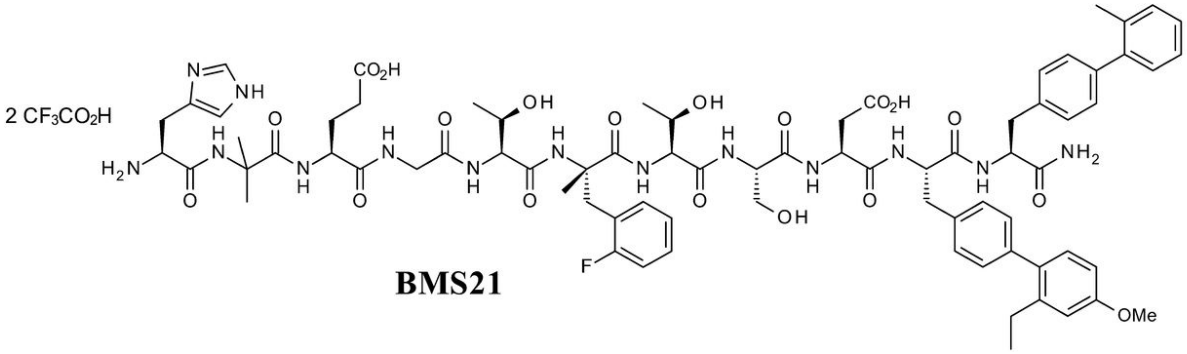
BETP



Compound 2



TT15 (Isomer 1)



BMS21

Figure 1.13 Structures of putative allosteric ligands of the GLP-1 receptor used in this study

1.10 GLP-1R allosteric modulation and biased signalling

1.10.1 The GLP-1R and allosteric modulation by small molecule ligands

To date, there are two reported and confirmed selective allosteric modulators of the GLP-1R; Compound 2 (Novo Nordisk) and BETP (Eli Lilly) (Figure 1.12) (Knudsen *et* al, 2007; Sloop *et al,* 2010). In studies using recombinant cell lines, Compound 2 was able to potentiate ligand affinity and efficacy (in cAMP assays) at the GLP-1R but it did so in a peptide selective (probe-dependent) manner, in that only oxyntomodulin was potentiated, with minimal potentiation of any GLP-1 peptide or exendin-4 (Koole *et al,* 2010). BETP also behaved as a probe-dependent allosteric modulator of the GLP-1R function with a similar profile in binding and cAMP to Compound 2; oxyntomodulin affinity and potency in cAMP assays was enhanced with minimal effect on other orthosteric peptide ligands (Wootten *et al*, 2012). In addition to displaying modulator properties, Compound 2 is also a robust agonist of cAMP accumulation, whereas BETP is less efficacious at this pathway (Koole *et al*, 2010; Wootten *et al*, 2012).

Quercetin and other hydroxy-flavonoids have also been shown to act allosterically at the GLP-1R (Koole *et al*, 2010; Wootten *et al*, 2011). These naturally occurring compounds are widely consumed in the human diet and do not display any agonism at the GLP-1R. However, they potentiate GLP-1 efficacy and potency in $_{i}Ca^{2+}$ mobilization in a probe-dependent manner (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Wootten et al. 2011). These studies revealed that hydroxyflavonols (such as quercetin) positively modulated receptor activity of high affinity orthosteric peptides (GLP-1(7-36)NH_2, GLP-1(7-37) and exendin-4), but not the lower affinity peptides (full length GLP-1 variants and oxyntomodulin).

1.10.2 Biased signalling at the GLP-1R

As mentioned previously biased signalling arises from the ability of distinct ligands to couple the receptor to distinct signalling pathways with different prominence. All GLP-1R peptide ligands preferentially activate cAMP over ERK1/2 phosphorylation and Ca^{2+} mobilisation, however the relative degree of bias varies (Koole *et al,* 2010). For example truncated GLP-1 peptides and exendin are strongly biased towards cAMP over ERK1/2 phosphorylation, whereas in comparison oxyntomodulin and the full length GLP-1 peptides are more weakly biased towards cAMP. Therefore, this provides the possibility that distinct agonists can direct cellular signalling with unique precision and specificity. There is also evidence that small molecule agonists of the GLP-1R are also biased ligands of GLP-1R function in comparison to GLP-1 (Wootten and Savage *et al*, 2013). Small molecules such as Compound 2 and BETP (that interact with the intracellular face of the GLP-1R) are heavily biased towards recruitment of β-arrestin regulatory proteins over G protein-mediated signalling compared to GLP-1 peptides (See thesis results chapter 1 for more details). In addition, small agonists such as Boc5 and BMS21 (that are thought to interact in the N-terminus/top of TM bundle/ECLs) were biased towards ERK1/2 phosphorylation and Ca^{2+} mobilisation compared to GLP-1 peptide ligands.

In addition to agonists, signalling bias can be engendered in orthosteric ligands by allosteric modulators. This behavior has been observed at the GLP-1R. As mentioned above, Compound 2 and BETP displayed probe dependent modulation of cAMP signalling at the GLP-1R, whereby only oxyntomodulin was potentiated. However, this potentiation of oxyntomodulin efficacy was observed in a pathway-dependent manner, with augmentation of cAMP signalling, while no effect on ERK1/2 phosphorylation or Ca^{2+} signalling was observed (Koole *et al*, 2010). In addition, hydroxyflavonols such as quercetin only potentiated the effects of high affinity

peptides in Ca^{2+} mobilisation studies, while having no effect on formation of cAMP or ERK1/2 phosphorylation (Koole *et al*, 2010, Wootten *et al*, 2011). These studies reveal that both agonists and allosteric modulators acting at the GLP-1R can exhibit stimulus bias, a phenomenon that provides the potential to develop ligands to fine tune physiological responses at the GLP-1R.

SECTION 3:

1.11 Diabetes mellitus (DM)

1.11.1 General Introduction

Diabetes mellitus (DM) is a disorder resulting from disruption of glucose homeostasis and can be classified into two types, Type 1 and Type 2 (T2). Type 1 DM involves an auto-immune response, whereby the body's immune system targets and destroys its own β-cells leading to eventual and almost complete abolishment of insulin biosynthesis and secretion. T2DM is primarily characterised by cellular resistance to the effects of insulin resulting in ineffective glucose storage (mainly by the peripheral tissues) and hyperglycemia (DeFronzo 1992). T2DM accounts for 90% of all diabetics and is one of the most rapid growing diseases worldwide, causing an increase in morbidity and mortality, affecting 170 million people worldwide, projected to grow to 360 million by 2030 (Wild *et al.*, 2004).

1.11.2 Pathophysiology of Type 2 DM

T2DM is characterised by dysfunction of both insulin synthesis and signalling and a subsequent progressive loss of β-cell mass and function due to the β-cells inability to secrete enough insulin to insulin resistance (Dalle et al. 2013). The resulting hyperglycaemia can have its own toxic effect on β-cells (Leahy *et al.*, 1986). In addition, there is severe resistance of insulin actions in

target tissue such as the liver and muscles causing elevated plasma glucose levels and ineffective glucose storage. Defective insulin signalling inevitably causes decreased glucose transport and phosphorylation, reduced glycogen synthesis, impaired glycolysis, and in addition glucose oxidation can further contribute to insulin resistance (DeFronzo & Tripathy 2009)

Co-morbidities include heart disease, stroke, neuropathy, blindness and kidney disease that occur as a consequence of hyperglycaemia. Recent evidence suggests that T2DM also increases the risk of developing dementias, such as Alzheimer's disease, with 85% of AD patients found to have diabetes or at least increased fasting glucose levels (Cole *et al.*, 2007; Holscher *et al.*, 2010).

T2DM is initially characterised by reduced insulin sensitivity in peripheral tissues; the pancreas responds by increasing insulin synthesis/secretion. As the disease progresses the constant demand for elevated insulin results in eventual decline of β-cell function/mass (β-cell exhaustion/glucose toxicity). There are multiple pathophysiologies associated with induction of T2DM. One of the largest risk factors is obesity, which is further associated with numerous complications such as cardiovascular disease, cancer, GI disease and arthritis. Importantly, obesity is the main modifiable risk factor for T2DM with 80% of people overweight when they are diagnosed, 40% of which are clinically obese.

Obese individuals develop resistance to the cellular actions of insulin, characterised by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle (Saltiel and Kahn 2001; Hribal et al. 2002). There are many mechanisms behind obesity-associated insulin resistance some of which include elevated fatty acids, inducing insulin resistance through intracellular metabolites that activate PKC, which in turn activate serine/threonine kinases that inhibit insulin signalling. There are also obesity-associated changes in secretion of adipokines that modulate insulin signalling, obesity-associated inflammatory

factors, and accumulated adipose tissue macrophages (ATMs) that increase adipose tissue production of inflammatory cytokines that inhibit insulin signalling (Kanda 2006; Weisberg et al. 2006; Lumeng et al. 2007). Endocrine and inflammatory mediators may converge on serine/threonine kinases to inhibit insulin signalling (Qatanani & Lazar 2007). Obesity-associated activation of NF-κB can heighten inflammatory responses that exacerbate insulin resistance. In addition, suppressors of cytokine signaling proteins (SOCS), associated with inflammation have been implicated to interfere with IRS-1 and IRS-2 tyrosine phosphorylation or by targeting them to proteosomal degradation leading to decreased insulin production (Rui et al. 2002; Ueki et al. 2004).

Hyperglycemia, classified by elevated levels of glucose concentrations in the blood, is known to exacerbate insulin resistance and β-cell dysfunction (Hosokawa et al. 1996). The effects of hyperglycemia have major detrimental physiological outcomes on the body; it predisposes the body to vascular dysfunction, retinopathy, blindness, renal disease; atherosclerosis, increased risk of heart attack, stroke and cardiovascular mortality.

In recent decades the prevalence of T2DM and associated obesity has escalated dramatically, which is largely attributed to lifestyle and is currently a severe global health burden. There have been consequential increases in morbidity and mortality associated with secondary complications of diabetes, such as cardiovascular diseases, kidney failure and retinopathy, which have all increased (Ashcroft & Rorsman 2012). It is therefore imperative that new approaches for disease prevention and treatment are identified.

1.11.3 Current treatments for T2DM

Until recently, drug treatments for T2DM have primarily focused on glycaemic control, through lowering blood glucose levels as well as haemoglobin A_{1C} (HbA_{1C}). However, there have been significant advances that have focused on delaying progression of glucose

intolerance and developing newer classes of blood glucose-lowering medications to supplement existing therapies. While current management for T2D continues to encompass traditional drugs such as thiazolidinediones (TZDs), metformin, and sulfonylureas (SUs) that focus on β-cell failure and/or insulin resistance, newer agents that target other defects such as incretin deficiency and resistance are increasingly used as conjunct therapies.

Sulfonylureas (SUs) were one of the first widely used anti-hyperglycaemic medications for the treatment of T2DM, triggering insulin release from pancreatic beta cells. SUs have antihyperglycemic effects by augmenting insulin secretion through closure of pancreatic K^+ channels. They reduce the risk of vascular dysfunction, however associated with weight gain. The greatest risk of SU treatment is severe hypoglycemia a result of SU-mediated insulin secretion independent of plasma glucose concentration (Kirpichnikov et al. 2002).

Second generation drugs have provided more effective treatments than SUs with fewer side effects. These include metformin, the most commonly prescribed drug on the market, acting as a potent insulin sensitizer in the liver with no associated weight gain. Metformin also decreases free fatty acid in plasma, decreases vascular dysfunction as well as decreasing insulin resistance and the proinflammatory response (Rendell 2004). Thiazolidineliones (TZDs) were introduced in the 1990s. This class of drugs provides not only potent insulin sensitisation in the liver and muscles but inhibits both the increased rate of hepatic gluconeogenesis and lipolysis, lowering plasma FFA levels. TZDs augment and maintain insulin secretion/insulin resistance (disposition) index in both drug-naïve and SU-treated T2DM patients. Compared to metformin and SUs, they also maintain long-term durability of glycaemic control, following an initial decline in HbA_{1c}, as a consequence of their β-cell protective function (Gastaldelli et al. 2013). Common adverse side effects associated with TZDs include weight gain and fluid retention (peripheral edema). TZDs

are mostly commonly used in combination with other glucose lowering agents such as metformin/SUs (Garber et al. 2007).

1.12 GLP-1R as a therapeutic

1.12.1 GLP-1R as a therapeutic target for T2DM

Recently it has been revealed that T2D does not develop without the onset of progressive β-cell failure (Halban et al. 2014). Studies have shown that neither metformin nor SUs provide any protection against loss of β-cell function (TODAY Study Group 2013; Maedler et al. 2005), thereby allowing disease progression that would eventually lead to insulin therapy. In addition, these compounds do not result in weight loss and therefore do not address the problem of obesity that often accompanies T2DM.

In the last decade, receptors that bind incretin hormones (GIP and GLP-1) have attracted significant interest as potential therapeutic targets, due to the their insulinotropic properties. In T2DM patients, the insulinotropic action of GIP is diminished, whereas that of GLP-1 is substantially preserved, although secretion of the latter appears to be diminished (Nauck et al. 1993; Vilsbøll et al. 2003). GLP-1 administration to these patients lowers blood glucose levels during fasting and after eating and is associated with weight loss, and decreased rate of gastric emptying, thereby promoting satiety and decreasing nutrient consumption. In addition, Zucker diabetic fatty (ZDF) rats displayed consistent increase in islet size, and a significant decrease in the number of apoptotic pancreatic β-cells following GLP-1 treatment. Thus, methods of enhancement of circulating concentrations of GLP-1 and/or GLP-1R signalling have been established as therapeutic strategies in T2DM.

GLP-1R agonists and dipeptidyl-peptidase-4 (DPPIV) inhibitors are now widely and

successfully used for this condition. DPPIV inhibitors augment endogenous active GLP-1 (in addition to GIP and oxyntomodulin) concentrations. These agents include saxagliptin, which increases circulating concentrations of native GLP-1 3-5 fold (Hjøllund et al. 2011), thereby resulting in increases in insulin secretion, decreases in gastric emptying, and decreases blood glucose levels and an inhibition in glucagon release.

Exenatide (Byetta ®), a synthetic version of exendin-4 has been approved by the FDA for the treatment of T2DM. Exenatide administrated parenterally reduces HbA_{1c}, increases insulin secretion and preserves β-cell function. Similar to GLP-1 it suppresses inappropriate glucagon secretion, slows postprandial gastric emptying and suppresses appetite with longer term effects on weight reduction (DeFronzo et al. 2005). Another incretin-based therapy approved more recently by the FDA is liraglutide (Victoza®), a longer-acting, once-daily human GLP-1 analogue. Like exenatide, liraglutide is resistant to DPPIV degradation (due to binding to plasma albumin) however, it has better gastrointestinal tolerance, and lower incidence of minor hypoglycemia.

Despite the promise of these incretin-based therapies, each is associated with side effect profiles. DPPIV inhibitors have been associated with upper respiratory tract infection, headache, and cough. They also have the potential to inhibit the cleavage other substrates besides GLP-1 (including hormones, neuropeptides, and chemokines) prolonging the action of neuropeptides such as substance P and macrophage-derived chemokines. That may produce inflammation (effect on substance P), increase blood pressure (effect on neuropeptide Y), or cause allergic reactions (effect on chemokines).

The GLP-1 mimetics liraglutide and exenatide have been associated with nausea and acute pancreatitis in several patients. Liraglutide has also been associated with thyroid C-cell

hyperplasia in rats (a precursor for thyroid cancer)(Drucker et al. 2010). In addition, GLP-1R analogues are peptides that are required to be administered by subcutaneous injection, which has low patient compliance compared to oral therapies. Exploration of alternative treatments, in particular small molecule allosteric ligands that target the GLP-1R are currently being pursued by the pharmaceutical industry as the ideal therapeutic approach to treatment of T2DM.

1.12.2 Future directions for GLP-1 based therapeutics and challenges facing discovery

The development of incretin-based therapies holds great promise as therapeutics for patients with T2DM. Future directions of incretin-based therapies aim to exhibit long-term favourable effects on β-cell mass and function. The use of small molecule allosteric modulators for the GLP-1R offers many advantages. One major advantage is their potential ability to be administrated orally, thereby increasing patient compliancy. Furthermore allosteric modulators have the potential to maintain spatial and temporal characteristics of endogenous signalling. Allosteric ligands modulate orthosteric ligand binding and/or efficacy through mediating changes in receptor conformation. Furthermore, these effects reach a limit governed by the cooperativity between the orthosteric an allosteric ligand providing the potential for preventing overdose. For these reason, allosteric modulators are being pursued as the ideal therapeutic for GLP-1R.

The phenomenon of biased signalling also holds great promise. The design of ligands (allosteric or orthosteric) that alter signal bias offers the potential of augmenting physiologically beneficial signalling pathways to the exclusion of detrimental ones and providing a novel mechanism to enhance the positive effects of activating the GLP-1R but further reduce unwanted side effect profiles (such as those seen with current GLP-1 mimetics). The key challenge, however, is that there is currently only a limited understanding of which signalling pathway or combination of

pathways are lead to the desired therapeutic outcome. The GLP-1R system is also complicated, in that there are at least six endogenous ligands for this receptor, therefore development of allosteric ligands may be challenging due to probe dependent effects of allosteric modulation. Therefore detailed understanding allosteric modulation, probe dependence and biased signalling at the GLP-1R will be crucial in order to rationally develop drugs to target this receptor that display improved therapeutic profiles.

CHAPTER 2:

Materials and Methods

2.1 Reagents

2.1.1 Peptide Ligands

GLP-1(7-36)NH_2, GLP-1(1-36)NH_2, exendin-4 and oxyntomodulin were purchased from American Peptides (Sunnyvale, CA, USA). GLP-1(9-36)NH_2, was purchased from Auspep Pty. Ltd.

2.1.2 Small Molecule Ligands

Small molecule GLP-1 ligands 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine (BETP) (Sloop et al., 2010), 1,3-bis [[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid (Boc5) (Chen et al., 2007), (2S-3-(4' - cyanobiphenyl-4-yl)-2-({[(8S)-3-{4-[(3,4-dichlorobenzyl)oxy]phenyl}-2-oxo-7- (phenylcarbonyl)-2,3,6,7,8,9-nexahydro-1H-[1,4]oxazino[3,2-G]isoquinolin-8-yl]carbonyl}amino)propanoic acid (TT15) (Rao, 2009) and BMS21 (Mapelli et al., 2009) were provided by Eli Lilly. 6.7-dichloro-2-methylsulfonyl-3- tert-butylaminoquinoxaline (Compound 2) (Knudsen et al., 2007) was generated according to a method published previously (Teng et al., 2007) to a purity of ~95%, and compound integrity was confirmed by NMR.

2.1.3 General Reagents

Dulbecco's Modified Eagle's Medium (DMEM), hygromycin B and Fluo-4 acetoxymethyl ester (Fluo-4 AM) and coelentrazine-h were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Thermo Electron Corporation (Melbourne, VIC, Australia).

AlphaScreen reagents, ^{125}I-labeled Bolton-Hunter reagent, 384-well Optiplates and Proxiplates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). SureFire^{TM} ERK1/2 reagents were obtained from TGR Biosciences (Adelaide, SA, Australia). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or BDH Merck (Melbourne, Victoria, Australia) and were of an analytical grade.

2.2 Cell culture

For basic cell culture techniques, refer to Freshney (1994).

2.2.1 Maintenance of Cells

Cell culture maintenance was performed in PS2 Laminar Flow Hoods (Email Air Handling, NSW, Australia), under sterile conditions. Cells were maintained in tissue culture flasks at 37° C in a CO_2 water jacket incubator (Forma Scientific, Oh, USA) at 5 % CO_2 and 85 % humidity.

Cells were grown in monolayer in the appropriate media. FlpIn Chinese hamster ovary (CHO) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) #11995-065, supplemented with 1 mM sodium pyruvate (110 mg/L), 25 mM D-glucose (4.5 g/L), 4 mM L-glutamine and fetal bovine serum (FBS; 10 % w/v) with or without hygromycin B (300 μg/mL). Cells were incubated in a humidified environment at 37° C in 5% CO_2. Once cells reached 80-90% confluency, media was removed, cells were washed once with sterilized phosphate buffered (PBS) and detached with versene and trypsin (0.01 % w/v). The cell suspension was removed and either seeded into a new flask to maintain the cell line, pelleted for cell counting and subsequent plating, or discarded.

2.2.2 Freezing and thawing cells

To freeze cell cultures, cell suspensions were pelleted for 3 min at 350 × g. The supernatant was

discarded and cells were resuspended in FBS supplemented with 10% dimethylsulphoxide (DMSO) and 400 µl of resuspended cells, at a density of 2×10^6 cells/ml were aliquoted into 0.5 mL cryrobank vials. Each cryobank vial was labeled with cell type, date frozen and passage number. Cell stocks were slowly frozen at -80° C and then transferred into liquid nitrogen for long-term storage.

To begin new cultures, frozen stocks were rapidly thawed at 37° C and gently pipetted into sterilized Falcon tube containing pre-warmed media. Cells were transferred to a centrifuge and spun for 3 min at 350 × g to pellet the cells. The supernatant was discarded and cells were gently resuspended in pre-warmed media and transferred into a sterile culture flask.

2.2.3 Counting cells

A haemocytometer and cover slip were cleaned with 80% ethanol, and the cover slip placed over the haemocytometer channels. The resuspended cells were diluted 10-fold in PBS with the addition of trypan blue (that stains dead cells). Cell suspensions were placed drop-wise on either side of the haemocytometer, allowing the liquid to be drawn into the haemocytometer via capillary action. Under a microscope, cells were counted in five squares and the average cell number per square determined. Cells stained blue from the trypan blue were excluded from the cell count. The average cell number per square was multiplied by the dilution factor (in most cases this was 10) and by $1x10^4$ to give cells/mL. The required number of cells was then dispensed into the new flask, dish or 96 well plate, at the required density and were replaced in the incubator.

2.2.4 Transient transfections of cells

Cells were seeded into 100 mm petri dishes and allowed to reach 60-70% confluency. Transient transfections were performed using polyethylenimine (PEI). Briefly, a solution containing DNA construct and PEI at a ratio of 1:6 DNA:PEI (5µg:30µl per 100 mm dish), was prepared in 150

mM NaCl (500 µl per 100 mm dish) and allowed to incubate at room temperature for 20 min. Following the incubation period, DNA transfection mix was added to dishes/flasks containing cells and incubated at 37° C overnight (24 h.). Media was removed and cells were harvested and seeded at appropriate densities into 96 well plates and further incubated at 37° C for another 24 h. Transiently transfected cells were assayed at 48 h. post transfection.

2.2.5 Generation of stable cell lines

FlpIn CHO cells were seeded into either, T25 cm^2 or T75 cm^2 sterilized culture flasks and allowed to reach 60-70% confluency. Stable transfections were performed using PEI. The PEI:DNA solutions were prepared as above. 24 h. post transfection, media was removed from cells and replaced with fresh media supplemented with HygroGold (600 µg/ml). Selection of stable colonies in these cells was usually achieved in 1-2 weeks with antibiotic selection.

2.3 Bioluminescence Resonance Energy Transfer

2.3.1 General Introduction

FlpInCHO cell lines stably expressing GLP-1R-Rluc8 and either βarrestin1- or βarrestin2-Venus (as well as GRK2,-3,-5, and -6) were generated using Gateway Technology as previously described (Willard, Wootten, et al. 2012b; Savage et al. 2013). Cells were seeded in 96-well white culture plates (CulturPlate-96, White Opaque 96-well Microplate, Sterile and TissueCulture Treated #6005680, PerkinElmer) at 4×10^4 cells/well in 100 µl Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) and incubated for 24 h. at 37° C, 5% CO_2. Following incubation, cells were rinsed once with 100 µl Hanks Balanced Salt Solution (HBSS) purchased from Invitrogen (Carlsbad, CA) to remove traces of phenol red and serum-starved in 80 µl of fresh HBSS supplemented with 0.1 % BSA for a further 30 min (37°C

humidifier). After serum-starvation, 10 µl of the Rluc substrate coelenterazine-h was added in low light conditions to reach a final concentration of 5 µM and incubated at 37°C for 5 min (light sensitive). Following incubation, the corresponding agonist was added and bioluminescence resonance energy transfer (BRET) readings were collected using a LumiSTAR Omega instrument (BMG LABTECH GmbH, Ortenberg, Germany), 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 min to determine the time at which βarrestin1 and βarrestin2 recruitment was maximal for each ligand in the absence and presence of BETP. Co-addition of ligands was performed for interaction assays and BRET signals were collected at this peak time point.

2.3.2 Construct generation:

Please refer to **(Savage et al. 2013),** presented in Chapter 4.

2.4 Plasmid Generation and Preparation

2.4.1 Transformation of vectors into $DH_{5\alpha}$ cells

All plasmids were transformed into competent $DH_{5\alpha}$ cells via heat shock. Briefly, DH5α cells were thawed on ice. Cells were gently mixed and 20 µl of competent cells were aliquotted into a chilled 1.7 ml Eppendorf tube. 1 µl of DNA was added to the cells (1 to 10 ng DNA), moving the

pipette through the cells while dispensing, and tapped gently to mix. Cells were incubated on ice for 30 minutes. Cells were then promptly heat-shocked for 45 seconds in a 42 °C water bath and placed immediately back on ice for 2 min. 600 µl of pre-warmed S.O.C. medium, Life Technologies (Cat. No. 15544-034) was added to cells and shaken at 170 rpm (37° C) for 1 hr. Cells were pelleted by centrifugation at 10,000 rpm for 1 min. 550 µl of S.O.C media was carefully discarded from the Eppendorf tube and the pellet was resuspended in the remaining 50 µl of media and spread onto agar plates with the appropriate selection antibiotics (100 µg/ml ampicillin, 50 µg/ml kanamycin, chloramphenicol 34 µg/ml) dependent on the plasmid. Plates were incubated overnight at 37 °C (approx. 16 h).

2.4.2 Colony selection

Single colonies that grew on the LB agar plates were selected, removed using a sterile 200µl pipette tip under aseptic conditions and miniprep cultures were prepared according to the QIAGEN® Plasmid Mini Kit (25) #12123 instructions (including Cell Suspension Solution, Cell Neutralization Solution, Lysis Buffer, Elution Buffer, and Mini Filter Tubes). Briefly, selected colonies were placed in a 15 ml Falcon tube containing 5 ml LB broth with the appropriate antibiotics. The cultures were incubated in an orbital shaker at 37° C for 16 hrs. Cells were pelleted (2 min, 10,000 × g, at room temperature), supernatant was discarded and cells resuspended in cell suspension solution. Lysis Buffer was added and cell lysis halted by the addition of chilled neutralization solution. Cell debris was pelleted (10 min 10,000 × g, at 4°C), and the supernatant containing the DNA was transferred to a Miniprep Filter Tubes contained within a collection tube. Centrifugation of the supernatant (1 min, 14,000 × g, at room temperature) allowed the filter to collect DNA. Flow-through was discarded; the filter tube connected to a sterile microcentrifuge tube, and DNA was eluted from the filter by the addition of Elution Buffer and centrifugation (30 sec, 14,000 × g, at room temperature). Miniprep DNA

was stored at -20°C. For long term storage, glycerol stocks were generated from overnight cultures of in 25 ml LB, the culture was then pelleted at 4000 rpm at 4°C and resuspended in 1 ml of 25% glycerol and stored at -80°C.

2.4.3 Amplification of DNA

Plasmid DNA was further amplified using either QIAGEN, HiSpeed® Plasmid Maxi Kit (25) #12663 or ORIGENE, PowerPrep™ HP Plasmid Maxiprep Kit #NP100009 (including Cell Suspension, Cell Lysis solution, Neutralization buffer, Column Equilibration and Wash buffers, Elution solution, Purification Resin and Maxi Columns). Briefly, glycerol stocks containing DNA of interest were inoculated in 150 ml of LB broth containing either; ampicillin (100μg/ml), kanamycin (50μg/ml) or chloramphenicol (35μg/ml) and placed in an orbital shaker at 37°C for 16 hrs. Cells were pelleted by centrifugation (10 min, 10,000 × g at 4°C). Supernatant was discarded and the cell pellet resuspended in Cell Suspension Solution (containing RNase A). Cells were then lysed by addition of Cell Lysis Solution, and lysis terminated by adding Neutralization Solution. Cell debris was pelleted (30 min 14,000 × g at 4°C) and the supernatant (containing the DNA) was transferred into a previously prepared equilibrated Maxiprep column (equilibration buffer). The solution was allowed to drain by gravity flow (flow through was discarded). The column was washed with wash buffer and allowed to drain by gravity flow (flow through discarded). Plasmid DNA was eluted with Elution Buffer by gravity flow and flow through was collected in sterile 50 ml Falcon tubes. Isopropanol was added to the eluate, mixed and centrifuged (14,000 × g at 4° C) for 30 min. The supernatant was carefully discarded and the plasmid DNA pellet was washed in 70 % ethanol and centrifuged (14,000 × g at 4° C) for 5 min. The ethanol was fully pipetted off and the pellet was set aside to air dry for 10 min. Purified DNA was dissolved in 500μl of Invitrogen, UltraPure™ DNase/RNase-Free Distilled Water #10977-023, transferred into fresh sterile 1.7 ml eppendorf tubes. The DNA concentration was

calculated by measuring absorbance on a UV spectrometer (Ultrospec 2000, Amersham Pharmacia Biotech, NJ, USA) at 260 and 280 nm, and stored at -20° C.

2.5 Radioligand Binding Assay

Flp-In-CHO GLP-1R 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, and radioligand binding carried out as previously described (Koole et al. 2011). For each cell line in all experiments, total binding was defined by 0.5 nM ^{125}I-exendin(9–39) alone, and nonspecific binding was defined by the additional inclusion of 1 uM exendin(9–39). For analysis, data are normalized to the specific binding for each individual experiment.

2.6 cAMP Accumulation Studies

All cAMP studies were performed using an AlphaScreen cAMP Assay Kit (PerkinElmer, Vic, Australia). Briefly, FlpInCHO-hGLP-1R cells were seeded at a density of 3x10^4 cells/well into 96-well culture plates and incubated overnight at 37° C in 5% CO_2. Growth media was replaced with stimulation buffer [phenol-free DMEM containing 0.1% (w/v) BSA and 1 mM 3-isobutyl-1-methylxanthine] and incubated for a further 1 h at 37° C in 5% CO_2. Cells were stimulated with increasing concentrations of peptide ligand alone, small molecule ligand alone, or simultaneously with increasing concentrations of allosteric ligand and peptide, and incubated for 30 min at 37° C in 5% CO_2. The reaction was terminated by rapid removal of the ligand-containing buffer and addition of 50 µl of ice-cold 100% ethanol. After ethanol evaporation, 75 µl of lysis buffer [0.1% (w/v) BSA, 0.3% (v/v) Tween 20, and 5 mM HEPES, pH 7.4] was

added, and 10 μl of lysate was transferred to a 384-well ProxiPlate (PerkinElmer Life and Analytical Sciences). 5 μl of acceptor bead mix (1.0% AlphaScreen cAMP acceptor beads diluted in stimulation buffer) and 15 μl of donor bead mix [0.3% AlphaScreen cAMP donor beads, 0.025% AlphaScreen cAMP biotinylated cAMP (133 units/ μl) diluted in stimulation buffer, and preincubated for a minimum of 30 min] were added in reduced lighting conditions. Plates were incubated at room temperature overnight before measurement of the fluorescence using a Fusion-Alpha plate reader (PerkinElmer Life and Analytical Sciences) with standard AlphaScreen settings. All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel.

2.7 ERK1/2 Phosphorylation Studies

All ERK1/2 phosphorylation studies were performed using the AlphaScreen *Surefire* ERK1/2 phosphorylation kit including AlphaScreen anti-ERK acceptor beads and streptavidin donor beads all purchased from PerkinElmer (Melbourne, Victoria). FlpInCHO-hGLP-1R cells were seeded at a density of $3x10^4$ cells/well into 96-well culture plates and incubated overnight at 37°C, with 5% CO_2 in 100 μl DMEM, supplemented with 10% FBS. The following morning, media was removed and replaced with either 80 μl or 90 μl (depending on drug treatment) serum free DMEM, and serum starved for 6 hrs. Initial ERK1/2 phosphorylation time course experiments were performed over 1 h to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by agonists. Cells were stimulated with peptide ligand and/or simultaneously with increasing concentrations of allosteric ligand for the time required to generate a maximal ERK1/2 phosphorylation response (generally between 5-10 min depending on the ligand used. Media was then removed from the plates and quickly replaced with 50 μl per/well of AlphaScreen *Surefire* lysis buffer (Vic, Australia). Plates were frozen at -20° C before ERK1/2 phosphorylation detection. Plates were thawed at room temperature and 5 μl of

lysates were added to each well of a 384-well white Optiplate assay plate. AlphaScreen® SureFire® p-ERK1/2 activation and reaction buffer were brought to room temperature. Activation buffer was diluted 7-fold in reaction buffer, acceptor and donor beads were diluted 70-fold in activation/reaction mix. 8 μl of activation/reaction bead mix was added to the lysates in the 384-well proxy plate and covered with TOPSeal-A 384, a clear adhesive sealing film (PerkinElmer, Vic, Australia). Following 1 hr incubation at 37° C, 5 % CO_2, plates were rested at room temperature for 15min to equilibrate back to room temperature. Plates were read on a Envision® Alpha-reader with excitation filter 485/20 and emission filter 535/25(wavelength in nm/bandwidth in nm). Data were normalized to the maximal 3 % FBS response, determined at 7 min (peak FBS response).

2.8 Ca^{2+} mobilization Studies

The Flexstation Ca^{2+} assay is a fluorimetric assay that quantifies the elevation of intracellular calcium concentration in response to receptor agonists and Ca^{2+} ionophores. It is a real-time, live cell *in vitro* assay utilising Ca^{2+}-sensitive fluorescent dye to report Ca^{2+} concentrations within the cell which are increased by release of Ca^{2+} from intracellular stores or extracellular Ca^{2+} influx.

FlpInCHO-hGLP-1R cells were seeded at a density of 3×10^4 cells/well into 96-well culture plates in DMEM media supplemented with 10 % FBS, and incubated overnight at 37°C with 5% CO_2. Following incubation, media was removed and cells were washed once with 200 μl pre warmed (37°C) Ca^{2+} assay buffer (150mM NaCl, 2.6mM KCl, 1.18mM $MgCl_2.H_2O$, 10mM D-glucose, 10 mM HEPES, 2.2mM $CaCl_2.2H_2O$, (0.5% w/v) BSA and 4mM probenecid (pH 7.4)). Once washed, 90 ul of Ca^{2+} assay buffer was added to each well supplemented with 1 μM Fluo-

4, AM, cell permeant #F-14217 (Invitrogen, Carlsbad, CA) in low light conditions (light sensitive). The plate was wrapped in aluminium foil and incubated for 1 hrs. at 37° C. Receptor-mediated intracellular Ca^{2+} mobilization was determined as described previously (Werry et al., 2005). For agonist assays (peptide and small molecule ligands), increasing concentrations of ligand were added in the FlexStation (Molecular Devices, Palo Alto, CA). For allosteric interactions (where little or no calcium agonism was observed by the allosteric compounds), increasing concentrations of allosteric modulators were added 30 min before addition of peptide agonist in the FlexStation. Fluorescence was determined immediately after ligand addition in the Flexstation, 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. Peak magnitude was calculated using five-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP.

2.9 Data analysis

2.9.1 Equations

All data obtained were analyzed in Prism 6.0 (GraphPad Software Inc., San Diego, CA). Concentration response signalling data were analyzed using a three-parameter logistic equation as described previously (May et al. 2007).

Equation (1)

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

Bottom represents the y value in the absence of ligand(s), Top represents the maximal stimulation in the presence of ligand/s, [A] is the molar concentration of ligand, and EC_{50} represents the molar concentration of ligand required to generate a response halfway between Top and Bottom. Likewise, this equation was used in inhibition binding, replacing EC_{50} with IC_{50}. In this case, Bottom defines the specific binding of the radioligand that is equivalent to nonspecific ligand binding, whereas Top defines radioligand binding in the absence of a competing ligand. In a similar manner, the IC_{50} value represents the molar concentration of ligand required to generate a response halfway between Top and Bottom.

An allosteric ternary complex model (May et al. 2007) was applied to binding inhibition concentration curves to determine ligand cooperativity. In this case, non-depletion of ligands was assumed (Avlani et al. 2008):

Equation (2)

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

where

Equation (3)

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

and 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 equilibrium dissociation constant for the radioligand, respectively. [B] and K_B denote the concentration of allosteric ligand and equilibrium dissociation constant for the allosteric ligand, respectively. [I] and K_I denote the concentration of peptide agonist used in competition with the radioligand and the equilibrium dissociation constant for the peptide agonist, respectively. α and α'represent cooperativity factors, which are measures of the magnitude and direction of the allosteric interaction between the modulator and the radioligand, or the peptide agonist, respectively. Values of $\alpha > 1$ are indicative of a modulator-mediated increase in binding activity, whereas values of α between 0 and 1 are indicative of a modulator-mediated decrease in binding affinity.

Interaction data from signalling assays were analyzed with an operational model of allosterism to determine cooperativity factors in efficacy:

Equation (4)

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

where E_{max} is the maximal possible response of the system (not the agonist), basal is the basal level of response in the absence of agonist, K_B denotes the functional equilibrium dissociation

constant of the agonist (B), τ is an index of the coupling efficiency (or efficacy) of the agonist and is defined as the total concentration of receptors divided by the concentration of agonist-receptor complex that yields half the maximum system response (E_{max}), and n is the slope of the transducer function that links occupancy to response. $\alpha\beta$ is the combined affinity-efficacy parameter that measures the magnitude and direction of the functional interaction between the modulator and peptide agonist.

To quantify signalling bias, which may be manifested either as selective affinity (K_A) and/or efficacy (τ) of an agonist for a given pathway, agonist concentration-response curves data were analyzed with an operational model (Gregory et al. 2007), but modified to directly estimate the ratio of τ/K_A, in a manner similar to that described by (Figueroa et al. 2009), for each pathway:

Equation (5)

$$\frac{E_{\max} \times (\tau/K_A)^n \times [B]^n}{[B]^n \times (\tau/K_A)^n + (1 + [B]/K_B)^n}$$

where all other parameters are as defined for eq. 4. All estimated parameters are expressed as logarithms (mean ± S.E.M.); where relevant, statistical analysis was performed by one-way analysis of variance and Dunnett's post test using GraphPad Prism 6.0, and statistical significance was accepted at $p < 0.05$.

CHAPTER 3:

Allosteric Modulation of Endogenous Metabolites as an Avenue for Drug Discovery

Denise Wootten*, Emilia E. Savage*, Celine Valant, Lauren T. May, Kyle W. Sloop, James Ficorilli, Aaron D. Showalter, Francis S. Willard, Arthur Christopoulos, and Patrick M. Sexton

Mol Pharmacol **82:** 281 - 290

*** Co-first Authors**

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter [insert chapter number]

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was involved in the design of the experiments and performed the majority of the in vitro cell signalling studies and the data analysis on these. I also assisted in the manuscript preparation.	50%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Denise Woottten	Development of ideas, contribution to writing and revision of manuscript	
Patrick Sexton	Development of ideas, manuscript preparation	
Celine Valant	Contribution towards experiments	
Lauren May	Contribution towards experiments	
Kyle Sloop	Contribution towards in vivo studies	
James Ficorilli	Contribution towards in vivo studies	
Aaron Showalter	Contribution towards in vivo studies	
Francis Willard	Contribution towards in vivo studies	
Arthur Christipoulos	Provision of expertise	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**05/02/2015**
Main Supervisor's Signature	[redacted]	**05/02/2015**

1521-0111/12/8202-281–290$25.00
MOLECULAR PHARMACOLOGY
Vol. 82, No. 2
79319/3783058

Mol Pharmacol 82:281–290, 2012

Allosteric Modulation of Endogenous Metabolites as an Avenue for Drug Discovery[S]

Denise Wootten, Emilia E. Savage, Celine Valant, Lauren T. May, Kyle W. Sloop, James Ficorilli, Aaron D. Showalter, Francis S. Willard, Arthur Christopoulos, and Patrick M. Sexton

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria, Australia (D.W., E.E.S., C.V., L.T.M., A.C., P.M.S.); and Endocrine Discovery (K.W.S., J.F., A.D.S.) and Translational Science and Technologies (F.S.W.), Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

Received April 13, 2012; accepted May 10, 2012

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

This work was supported by the National Health and Medical Research Council of Australia [Project Grants 1002180, 519461] and the Australian Research Council [Discovery Grant 110100687].
D.W. and E.E.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.112.079319.
[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

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-1*H*-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-1*H*-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.

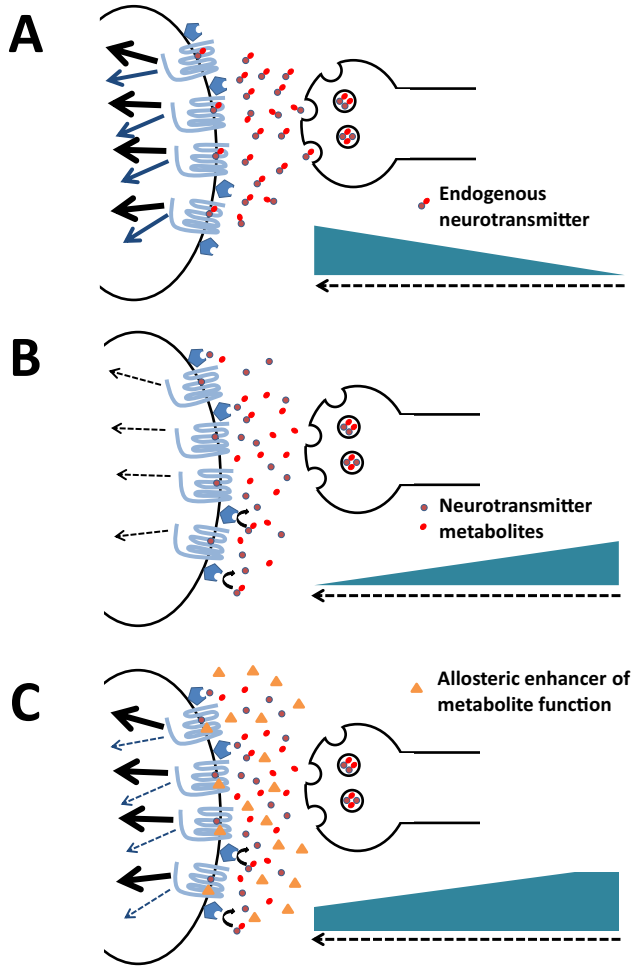


Fig. 1. Allosteric enhancement of metabolite activity as a novel mechanism of drug action. A, schematic illustration of neurotransmitter release and activation of postsynaptic receptors to elicit physiological signaling. B, the neurotransmitter is rapidly degraded by metabolizing enzymes, leading to 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.

$$E = \frac{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + [B]K_A + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \quad (1)$$

$$E = \frac{E_m(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]EC_{50})^n}{EC_{50}{}^n(K_B + [B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]EC_{50})^n} \quad (2)$$

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)NH_2	ACh	Adenosine	GLP-1(9–36)NH_2	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)		
Ca^{2+}	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)		
Ca^{2+}	N.D.			N.D.		
M_2 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)	
A_1-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.

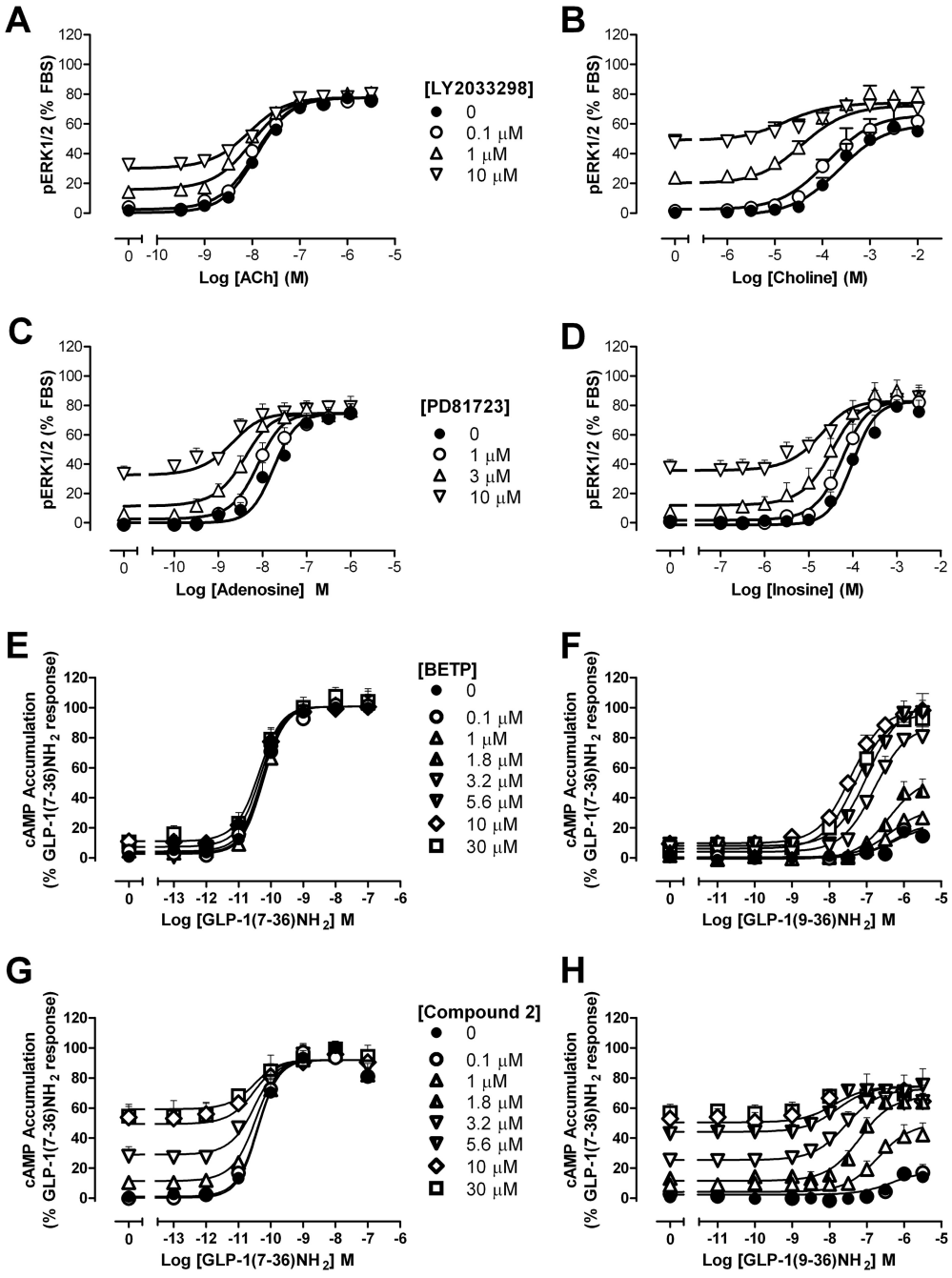


Fig. 2. Small-molecule ligands of three different GPCRs display a high degree of positive allosteric modulation of the metabolite of the cognate ligand in intact cells. Interaction studies were performed in ERK1/2 phosphorylation assays between LY2033298 and ACh (A) or Ch (B) in FlpInCHO cells stably 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 ± 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

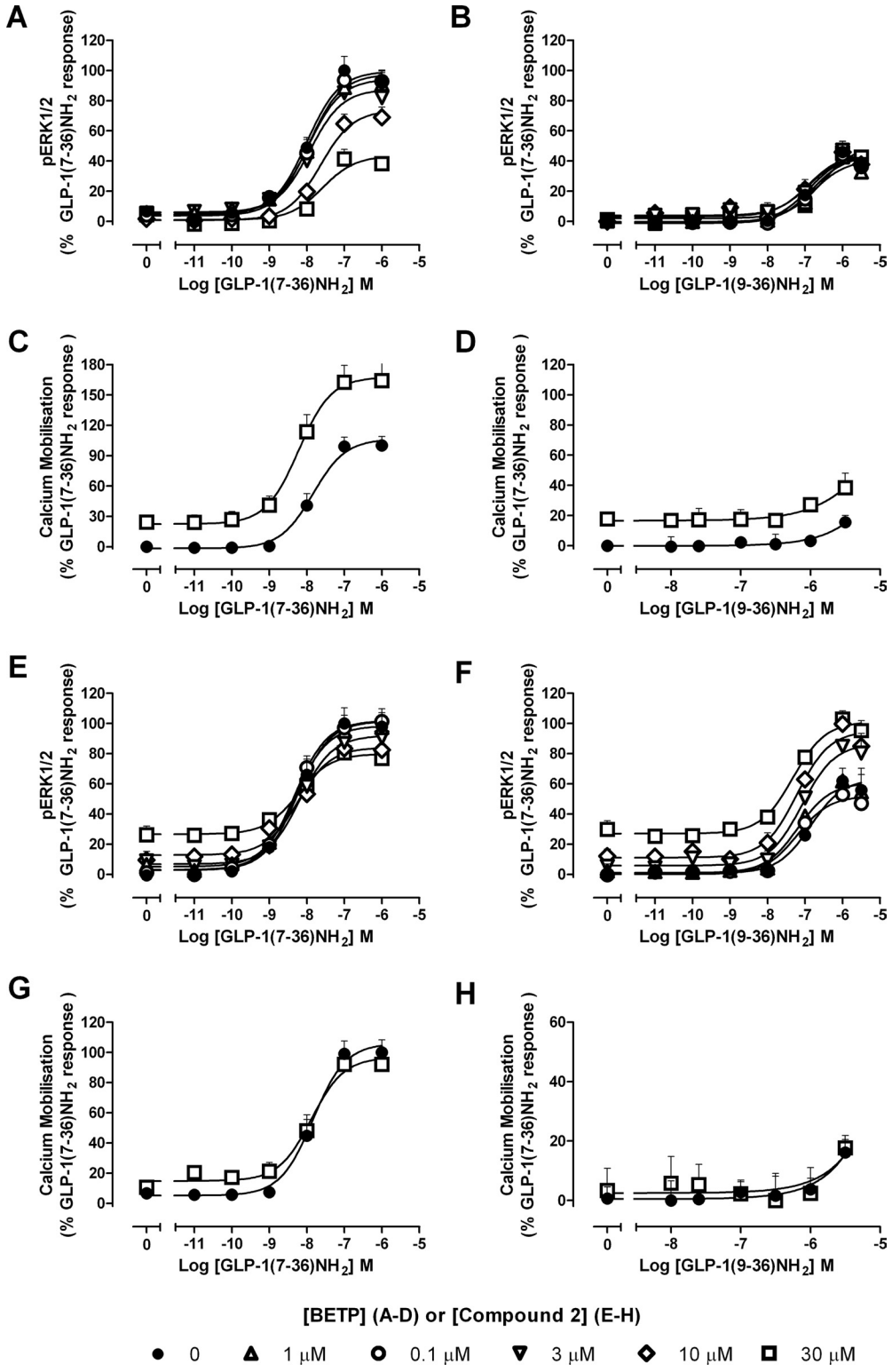


Fig. 3. Differing degrees of allosteric modulation of GLP-1(7–36)NH_2 and GLP-1(9–36)NH_2 by BETP or compound 2 at the GLP-1R in ERK1/2 phosphorylation and intracellular calcium mobilization in intact cells. Interaction studies between BETP (A–D) and GLP-1(7–36)NH_2 (A and C) or GLP-1(9–36)NH_2 (B and 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-

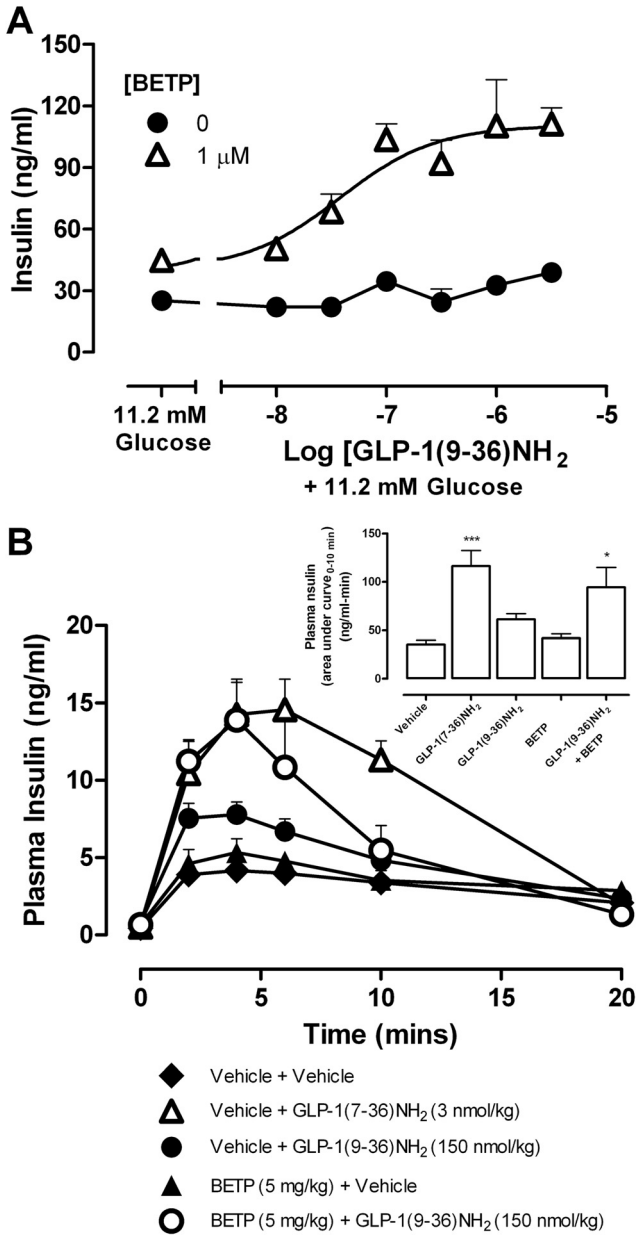


Fig. 4. Ex vivo and in vivo studies reveal allosteric modulation of the GLP-1 metabolite at the GLP-1R leads to insulin secretion. A, insulin concentrations from cultures of Sprague-Dawley rat islets incubated in medium containing high glucose (11.2 mM) and BETP with increasing concentrations of GLP-1(9–36)NH_2. 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 $G\alpha_i$-coupled M_2 mAChR and A_1-AR and cAMP for $G\alpha_s$-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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Address correspondence to: Prof. Patrick M. Sexton, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. [redacted]

CHAPTER 4:

A Simple Method to Generate Stable Cell Lines for the Analysis of Transient Protein-Protein Interactions

Emilia Elizabeth Savage, Denise Wootten, Arthur Christopoulos, Patrick Michael Sexton and Sebastian George Barton Furness

BioTechniques **54:** 217 - 221

PART B: Suggested Declaration for Thesis Chapter

[This declaration to be completed for each conjointly authored publication and to be placed at the start of the thesis chapter in which the publication appears.]

Monash University

Declaration for Thesis Chapter [insert chapter number]

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
I was involved in the development of the study, I designed and performed all the experiments presented and performed all the data analysis. I also contributed to the writing and revision of manuscript.	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Sebastian Furness	Development of ideas, contribution to writing and revision of manuscript	
Denise Wootten	Development of ideas, contribution to writing and revision of manuscript	
Arthur Christopoulos **Patrick Sexton**	Development of ideas and manuscript preparation	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**05/02/2015**
Main Supervisor's Signature	[redacted]	**05/02/2015**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Benchmarks

A simple method to generate stable cell lines for the analysis of transient protein-protein interactions

Emilia Elizabeth Savage, Denise Wootten, Arthur Christopoulos, Patrick Michael Sexton and Sebastian George Barton Furness
Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

BioTechniques 54:217-221 (April 2013) doi 10.2144/000114013
Keywords: BRET; flpIN; Gateway; protein interaction; bicistronic vector; arrestin; GPCR

Transient protein-protein interactions form the basis of signal transduction pathways in addition to many other biological processes. One tool for studying these interactions is bioluminescence resonance energy transfer (BRET). This technique has been widely applied to study signaling pathways, in particular those initiated by G protein-coupled receptors (GPCRs). These assays are routinely performed using transient transfection, a technique that has limitations in terms of assay cost and variability, overexpression of interacting proteins, vector uptake limited to cycling cells, and non-homogenous expression across cells within the assay. To address these issues, we developed bicistronic vectors for use with Life Technology's Gateway and flpIN systems. These vectors provide a means to generate isogenic cell lines for comparison of interacting proteins. They have the advantage of stable, single copy, isogenic, homogeneous expression with low inter-assay variation. We demonstrate their utility by assessing ligand-induced interactions between GPCRs and arrestin proteins.

Bioluminescence Resonance Energy Transfer (BRET) is a popular method for monitoring transient protein-protein interactions in live cells. It has been widely applied to study interactions between G protein-coupled receptors (GPCRs) and their interacting proteins such as G proteins, arrestins, G protein-coupled receptor kinases (GRKs) and other GPCRs (1,2).

This assay relies on the fusion of genetically encoded *Renilla* luciferase (RLuc) donor and green fluorescent protein (GFP) acceptor proteins to the interacting partners. To monitor interactions, cDNA chimeras encoding interacting partners (fused with donor and acceptor) are routinely prepared in separate plasmids and transiently co-transfected prior to the assay. Transient transfection assays can exhibit wide inter-assay variation due to variable transfection efficiency and may be costly in high-throughput formats, depending on the transfection reagent used. Transient transfections also typically result in very high transgene expression, potentially leading to a high baseline BRET signal or a low signal-to-noise ratio in ligand-induced BRET due to a high level of non-specific (collisional) interactions. In addition, overexpression can significantly alter the pharmacological behavior of receptors. During transient transfection, only a subpopulation of cells are transfected and there is a significant cell-cycle bias for DNA uptake, which has the potential to skew results of interaction studies (3).

To overcome the limitations of transient transfection and establish a reliable method for isogenic expression of interacting proteins, we designed bicistronic BRET vectors that take advantage of Life Technologies' (Carlsbad, California, USA) Gateway cloning and flpIN cell line systems. These vectors are based on the pEF5/FRT/V5-DEST flpIN destination vector from Life Technologies. This vector yields stable incorporation into a single (isogenic) site in the genome of flpIN cell lines. The *EF1*α promoter that drives expression of the bicistronic transcript is mammalian, rather than viral in origin, and provides stable expression. The encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) was chosen as this has been demonstrated to harbour true IRES activity in mammalian cells (4). It was placed upstream of the acceptor cDNA fusion as it has been shown to drive 7 to 10-fold greater expression of the second cistron (5). Thus the acceptor fusion will be in excess compared to the donor, minimizing the bystander BRET effect (6). We call these vectors BIVISTI for BRET IRES vector for isogenic stable incorporation to monitor transient interaction.

The parental pEF5/FRT/V5-DEST vector contains a V5 epitope tag downstream of the second recombination site and is flanked by *Bst*BI and *Pme*I restriction sites. We designed an

Method summary:

Here we present a series of bicistronic vectors based on the Gateway and flpIN systems which enable the rapid generation of isogenic cell lines for protein-protein interaction assays. As proof of principle, we assess ligand-induced interactions between G protein-coupled receptors and arrestin proteins generated via isogenic cell lines.

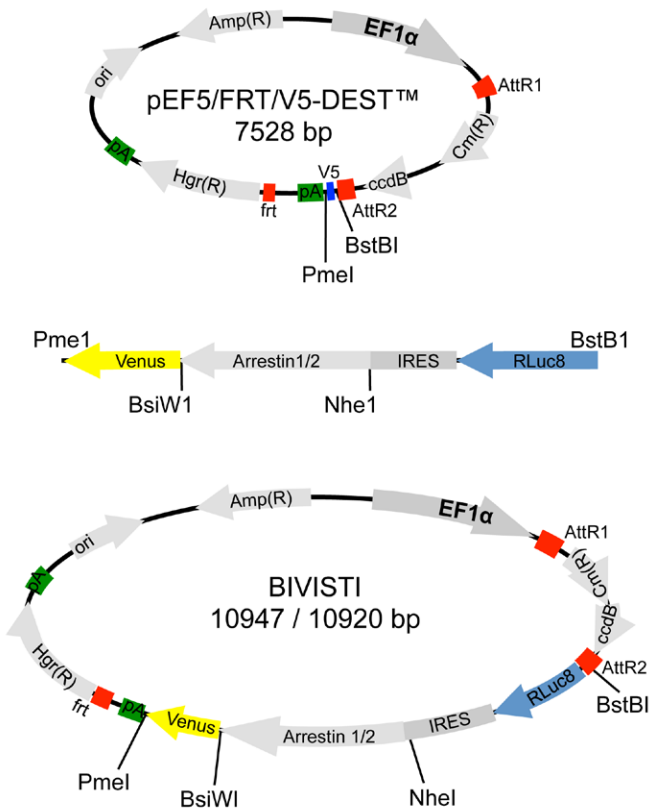

Figure 1. Scheme for replacement of the V5 epitope tag of parental vector pEF5/FRT/V5-DEST with the bicistronic BRET insert to generate a BIVISTI vector. 5' *Bst*BI and 3' *Pme*I sites corresponding to those flanking the V5 epitope flank the bicistronic insert. The bicistronic insert comprises the *Renilla* luciferase variant *RLuc8* followed by the ECMV IRES sequence, β-arrestin 1 or 2 fused to the GFP variant Venus. Key vector features are indicated using standard contractions.

insert flanked by *Bst*BI and *Pme*I sites that would replace this sequence with one containing a cassette with the coding sequence for *RLuc8* (GenBank: EF446136.1) followed by the unattenuated EMCV IRES sequence (nucleotides 149–713 relative to the polyprotein start site of GenBank: DQ288856) and a coding sequence for either *ARRB1* (β-arrestin 1, GenBank: NM_004041.3) or *ARRB2* (β-arrestin 2, GenBank: NM_004313.3) in frame with the GFP variant Venus (GenBank: DQ092360). The *RLuc8* sequence was placed in reading frame B, relative to the Gateway cassette, yielding a 26 amino acid linker when used with a stop codon-deleted coding sequence from a Gateway entry vector. The linker sequence is: DPAFLYKVVDIQHSGGRSSLEGPRFE and is predicted to form a mixture of extended and coil secondary structure (7). The native start codon from the EMCV IRES was retained, followed by an *Nhe*I site to allow conventional cloning of the acceptor fusion partner then the start- and stop codon-deleted coding sequence of *ARRB1* or *2* followed by a *Bsi*WI site and start codon-deleted coding sequence of Venus (Figure 1).

To validate these vectors, stop codon-deleted sequences for 3 distantly related GPCRs, GLP1R (glucagon-like peptide 1 receptor), CHRM1 (muscarinic 1, acetylcholine receptor), and AVPR2 (vasopressin 2 receptor) were cloned into pENTR11 and subsequently into the BIVISTI vectors by Gateway cloning, with GLP1R having an N-terminal cMyc epitope tag immediately following the signal peptide (8). Stable flpIN CHO cell lines were established by standard methods using these vectors. We then assayed these receptors for G protein-dependent function in calcium mobilisation or cAMP accumulation assays, confirming pharmacology consistent with untagged receptors in this cell background (an example of this is shown in Figure 2E). We then assessed the ability of these constructs to report transient recruitment of arrestin proteins to these receptors by ligand-induced BRET as described previously (9). We performed 3–4 independent time course experiments on stably transfected cells of various passage numbers ranging from 17 to 35. In addition, parallel transient transfection using the AVPR2/ARRB2 BIVISTI construct was performed. BRET readings were collected using a LUMIstar Omega instrument (BMG Labtech Ortenberg, Germany) 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 (ligand-induced BRET). This background-subtracted mean data from these 3–4 experiments are shown in Figure 2. In response to stimulation by 1μM arginine vasopressin of the AVPR2/ARRB2 cell line we saw a mean ligand-induced increase of 87 ± 3.2 milliBRET units (Figure 2A). This was slightly larger than the ligand-induced increase observed in parallel transient transfection of AVPR2/ARRB2 (71 ± 4 milliBRET), which also showed more point to point variability. Stimulation of the CHRM1/ARRB2 cell line with 100μM acetylcholine produced a mean ligand-induced increase of 16.2 ± 3.3 milliBRET (Figure 2A and B). In response to stimulation of the GLP1R/ARRB2 cell line with 100nM GLP-1(7–36)NH2 we saw a mean ligand-induced increase of 24 ± 2.1 milliBRET (Figure 2A and C). The ligand-induced change in milliBRET units for GLP1R is relatively small in comparison with the strongly coupled AVPR2; however, the response is highly consistent with small errors, allowing for the construction of a concentration response curve for the GLP1R/ARRB2 cell line in response to GLP-1(7–36)NH2 (Figure 2D). This concentration response curve was generated from the peak ligand-induced BRET values from 4 independent experiments and was fitted to a sigmoid dose-response curve using PRISM (GraphPad Software, La Jolla, California, USA) to yield a pEC_{50} for ARRB2 recruitment of 7.5 ± 0.1 with an R^2 of 0.92 (Figure 2D). To examine our ability to detect differences in arrestin recruitment patterns, we generated CHRM1/ARRB1 and GLP1R/ARRB1 stably transfected cell lines. In contrast to the CHRM1/ARRB2 cell line, there was no acetylcholine-dependent recruitment of ARRB1 to CHRM1, although GLP-1(7–36)NH2-dependent recruitment of ARRB1 to GLP1R was observed with a maximum ligand-induced BRET increase of 24 ± 3 milliBRET units (Figure 2F). To examine the correlation between receptor and β-arrestin expression, the GLP1R/ARRB2 was subjected to flow cytometry. Briefly, cells were harvested in versene (PBS + 0.5mM EDTA) and stained using AF647 (Life Technologies)-conjugated 9E10 (monoclonal against the cMyc epitope, produced in-house by standard methods, with degree of labeling = 4.6) at 2ug/μL and Sytox blue (Life Technologies) for live/dead discrimination. Data were collected on a FACSCantoII (BD Biosciences, San Jose, California, USA) and analyzed using FlowJo (Tree Star, Ashland, Oregon, USA). As a control, flpIN CHO cells expressing untagged GLP1R were stained and analyzed

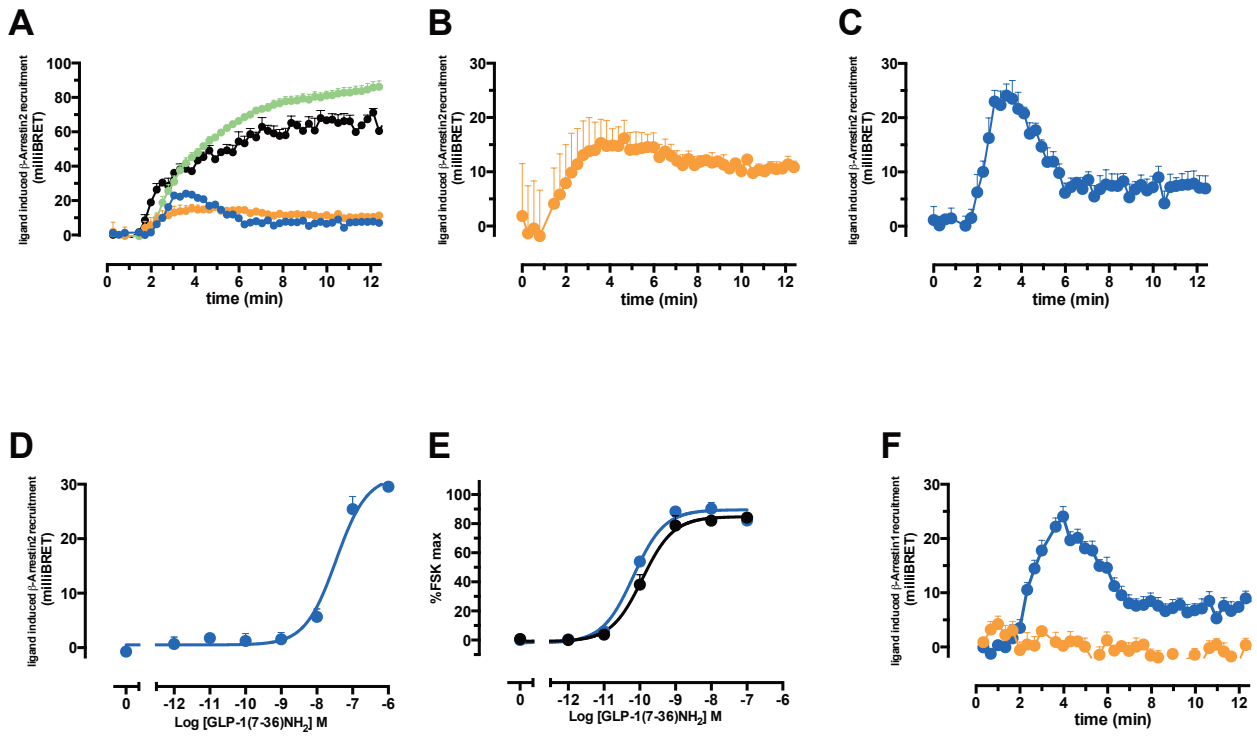

Figure 2. Ligand-induced recruitment of ARRB2 & ARRB1 to AVPR2, GLP1R and CHRM1 receptors. (A), Stable flpIN CHO cell lines expressing AVPR2-RLuc8/ARRB2-Venus (green), CHRM1-RLuc8/ARRB2-Venus (orange), GLP1R-Rluc8/ARRB2-Venus (blue) or flpIN CHO cells transiently transfected with AVPR2/ARRB2-Venus (black) and stimulated with 1μM arginine vasopressin for AVPR2-RLuc8/ARRB2-Venus, 100μM acetylcholine for CHRM1-RLuc8/ARRB2-Venus or 100nM GLP-1(7–36)NH2 for GLP1R-Rluc8/ARRB2-Venus**.** Data shown are mean ± SEM from 3–4 independent experiments performed in triplicate over passages 17 to 35. Peak ligand induced milliBRET responses for ARRB2 recruitment are 87 ± 3.2 (AVPR2), 16.2 ± 3.3 (CHRM1), 24 ± 2.1 (GLP1R), and 71 ± 4 (AVPR2 transient), respectively. (B) and (C) are the same as A but with expanded y-axis shown for CHRM1-RLuc8/ARRB2-Venus (B) and GLP1R-Rluc8/ARRB2-Venus (C)**.** (D), concentration response curve for GLP-1(7–36)NH2 induced recruitment of ARRB2 in the stable flpIN CHO, GLP1R-Rluc8/ARRB2-Venus cell line. Data are fit to the three-parameter logistic equation. The R^2 for the curve fit is 0.92 with a calculated pEC_{50} of 7.5 ± 0.1. Data are the mean ± SEM of four independent experiments conducted in triplicate. (E), comparison of concentration response curve for GLP-1(7–36)NH2 stimulated cAMP accumulation in GLP1R (black) and GLP1R-Rluc8/ARRB2 (blue) flpIN CHO stable cell lines. Data are normalized to the maximum cellular response to forskolin (%FSK max) and fit to the three-parameter logistic equation. The pEC_{50} values are 9.9 ± 0.1 and 10.2 ± 0.1. Data are the mean ± SEM of four independent experiments conducted in triplicate. (F), stable flpIN CHO cell lines expressing CHRM1-RLuc8/ARRB1-Venus (orange) or GLP1R-Rluc8/ARRB1-Venus (blue) were respectively stimulated with 100μM acetylcholine or 100nM GLP-1(7–36)NH2. Data shown are mean ± SEM from 3–4 independent experiments performed in triplicate over passages 17 to 35. No ligand induced ARRB1 recruitment is evident for CHRM1, whereas GLP1R showed a peak recruitment of are 24 ± 3 milliBRET in response to 100nM GLP-1(7–26)NH2.

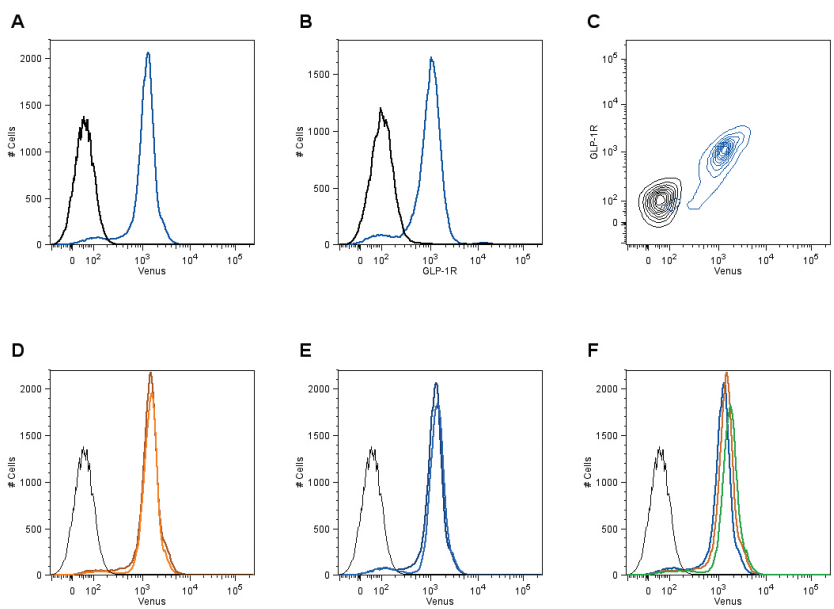

Figure 3. Flow cytometric analysis of GLP1R and ARRB2-Venus expression in stable flpIN CHO cells. Untagged GLP1R (black) and cMycGLP1R-Rluc8/ARRB1-Venus (blue) flpIN CHO cells were stained with AF647–9E10 and Sytox blue and live cells analyzed for Venus and cMycGLP-1R expression [(A), (B) and (C)]. (A) and (B) are histograms of relative fluorescence intensity of Venus and AF647–9E10, respectively, with the density plot in (C) showing relative fluorescence intensity of Venus plotted against that of AF647–9E10. (D) is a histogram of the relative fluorescence intensity of Venus from the CHRM1/ARRB2 flpIN CHO cell line at passage 17 (light orange) and 35 (dark orange) with stained, untagged GLP1R flpIN CHO as the control (black). (E) is a histogram of the relative fluorescence intensity of Venus from the GLP1R/ARRB2 flpIN CHO cell line at passage 17 (light blue) and 35 (dark blue) with stained, untagged GLP1R flpIN CHO as the control (black). (F) is a histogram of the relative fluorescence intensity of Venus from the AVPR2/ARRB2 (green), CHRM1/ARRB2 (orange) and GLP1R/ARRB2 (blue) flpIN CHO cell lines with stained, untagged GLP1R flpIN CHO as the control (black).

in parallel. Figure 3 shows the distribution of expression as a histogram plot for direct fluorescence from the Venus-tagged ARRB2 (Figure 3A, blue) and cMyc-tagged GLP1R-RLuc8 (Figure 3B, blue). The direct correlation of ARRB2 and GLP1R expression is demonstrated in the contour plot in Figure 3C (blue). The stability of expression over time was assessed by analysis of Venus fluorescence of the CHRM1/ARRB2 and GLP1R/ARRB2 cell lines at passage 17 and 35 (Figure 3D, orange and E, blue). A comparison of ARRB2-Venus expression between AVPR2/ARRB2 (green), CHRM1/ARRB2 (orange), and GLP1R/ARRB2 (blue) cell lines was also performed with stained untagged GLP1R cells as a control (black)(Figure 3F). Consistent with previous reports including Reference 10, stably transfected flpIN CHO cell lines show a narrow, single mode distribution of transgene expression. These plots are representative of three independent experiments.

In conclusion, we report a simplified scheme for generation of stable cell lines expressing both donor and acceptor fusion partners for protein-protein interaction studies by BRET. The incorporation of Gateway technology for the donor fusion makes this a useful tool for the development of cell lines for screening GPCRs and GPCR small molecule libraries for interactions with arrestin and other partner proteins. In addition, we have confirmed that this system also works with the distantly related Class C GPCR calcium sensing receptor (CaSR). The *Nhe*I and *Bsi*WI sites flanking the acceptor protein allow facile replacement of these fusions. We have developed BIVISTI variants containing GRK acceptor fusions and have been able to demonstrate robust recruitment of four isoforms of these kinases to GLP1R. The fact that cell lines generated using this system are isogenic also makes it a useful tool to examine structure-activity relationships of receptor mutants for partner protein interactions. This is a simple and robust system that should be amenable to a wide variety of applications and will assist in improving assay-to-assay variation as well as the general shortcomings associated with transient transfection.

Acknowledgements

PMS and AC are Principal Research Fellows of the National Health and Medical Research Council of Australia (NHMRC). This work was funded by the NHMRC program grant #519461

Competing interests

The authors declare no competing interests.

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Received: 16 October 2012; accepted: 27 February 2013

Address correspondence to Sebastian George Barton Furness, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia. [redacted]

CHAPTER 5:

Ligand mediated β-Arrestin interactions, allostery and biased signalling at the GLP-1R

Introduction

Different ligands acting at the same GPCR can stabilize multiple distinct receptor conformations linked to different functional outcomes. This phenomenon has been referred to as stimulus bias, biased agonism or functional selectivity and provides an opportunity to separate on-target therapeutic effects from adverse side effects through the design of drugs that show pathway selectivity (Violin & Lefkowitz 2007; Shonberg et al. 2014) (Drucker et al. 1987; Violin & Lefkowitz 2007; Holz et al. 1993; Kenakin 2011). Furthermore, this phenomenon extends to allosteric molecules; such that target GPCRs by binding to allosteric sites on the selected receptor (Baggio & Drucker 2007; Christopoulos & Kenakin 2002), have the potential to display biased agonism (allosteric agonists). In addition, allosteric modulators can alter the response imparted by the receptor following binding of an orthosteric ligand. Simultaneous binding of both an allosteric and orthosteric ligand in essence has the potential to create a 'new' version of the activated GPCR with its own set of functional properties. Thus, allosteric ligands can introduce a new dimension into pharmacological responses by modifying the affinity or the signal bias that the orthosteric ligand imparts on the receptor. They may also display probe dependence, in that the effect exerted by an allosteric modulator can vary depending on the co-bound orthosteric ligand (Sloop et al. 2010; Leach et al. 2007).

The canonical view of signalling by activated GPCRs is G protein-mediated, thus leading to generation of second messengers, one of which includes activation of cAMP generated via the adenylate cyclase system and another involving activation of Ca^{2+} mobilisation via the IP_3 pathway. G protein-mediated signalling can also result in activation of other signalling effectors such as MAPKs like pERK1/2.

In the classical paradigm, G protein-mediated signal transduction is terminated by the major GPCR regulatory pathway that involves the binding of β-arrestin proteins to phosphorylated

receptors. Many arrestin-bound receptors are inhibited from activating downstream G protein pathways, and internalisation and desensitisation of receptor signal. The canonical view is therefore that arrestin molecules are terminators of G protein-mediated signalling. However, in recent years arrestins have been identified as promoters of intracellular signalling independently of G protein activation. They mediate this signalling by acting as scaffolding proteins, recruiting their own subsets of signalling proteins that can be activated downstream of GPCR activation. Therefore, GPCR function can be mediated via G protein-dependent mechanisms or through G protein-independent mechanisms, such as those involving β-arrestin (Knudsen et al. 2007; Violin & Lefkowitz 2007).

Upon binding to the GLP-1R, orthosteric peptide ligands exert their effects by stabilizing active conformations of the receptor that predominantly couple the receptor to Gαs G proteins, stimulating the enzymatic activity of AC and thus favoring the formation of cAMP (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Drucker et al. 1987; Willard, Wootten, et al. 2012b; Holz et al. 1993). The GLP-1R is a complex system, with multiple endogenous and clinically used peptide ligands that exhibit different signalling profiles. GLP-1(7-36)NH_2 is the predominant endogenous ligand and activates multiple signalling pathways, such as cAMP, Ca^{2+} mobilisation and pERK1/2 (Sonoda et al. 2008; Baggio & Drucker 2007; Jorgensen et al. 2007). Oxyntomodulin and GLP-1(1-36)NH_2 are also found endogenously, and exendin-4 is used clinically; each of these peptides have also been observed to signal via multiple intracellular pathways. The selective GLP-1R small molecule ligands, BETP (Sloop et al. 2010) and Compound 2 (Knudsen et al. 2007) also display pleiotropic signalling, however neither of these small molecules fully mimic the actions of the native peptide ligand GLP-1(7-36)NH_2. These allosteric ligands can behave as agonists (in some signalling pathways) and also display probe dependence in their ability to modulate orthosteric

peptide responses. They induce an 18- to 25-fold potentiation of the affinity of bound oxyntomodulin, while having little effect on the other endogenous GLP-1R ligands or exogenous exendin-4 (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Willard, Wootten, et al. 2012b). Furthermore, these compounds induce biased signalling in the oxyntomodulin response, through potentiation of cAMP accumulation and insulin secretion, while having no effect on intracellular Ca^{2+} mobilisation or pERK1/2. BETP positively modulates exendin-mediated Ca^{2+} mobilization while negatively modulating pERK1/2 signalling for both GLP-1(7-36)NH_2 and exendin-4.

The GLP-1R is a promising target in pharmaceutical intervention for the treatment of type II diabetes. GLP-1 stimulates glucose-dependent insulin secretion from β-cells of the pancreas, as well as improving rodent β-cell mass by promoting proliferation and inhibiting apoptosis. While GLP-1R mediated cAMP, Ca^{2+} mobilisation and pERK1/2 signalling pathways have been associated with insulin secretion, more recent studies have also identified insulin secretion to be at least partly mediated by β-arrestins; in particular, β-arrestin1. In these studies, β-arrestin1 knockdown markedly decreased cAMP accumulation, impairing the function of PKA and other PKA-independent targets, including Epac, which resulted in decreased insulin secretion (Sonoda et al. 2008; Jorgensen et al. 2007). Furthermore, β-arrestin1 enhanced IRS-2 expression and the phosphorylation of both CREB and pERK1/2, while also mediating antiapoptotic behaviour effect of GLP-1 in β-cells by boosting GLP-1-mediated phosphorylation of the Bcl-2-associated death promoter protein (Bad). This phosphorylation of Bad at Ser112 is dependent on β-arrestin1-mediated pERK1/2 activation and favors β-cell survival (Violin & Lefkowitz 2007; Quoyer et al. 2010; Kenakin 2011; Feng et al. 2010). Most recently, β-arrestin1 also facilitated recruitment of c-Src to the agonist occupied receptor, which may underlie the proliferative actions of GLP-1 (Christopoulos & Kenakin 2002; Talbot et al. 2012).

To date, these studies on the involvement of β-arrestin1 have only been using the predominant physiological agonist GLP-1(7-36)NH_2. Given the studies showing stimulus bias by both GLP-1 endogenous and exogenous ligands, and the need to develop small molecule ligands to target this receptor, it is important to understand ligand-induced signalling bias at all signalling pathways for which the receptor can activate. In this study, β-arrestin1 and 2 recruitment to the GLP-1R was assessed in response to various endogenous and exogenous peptides, as well as several small molecule GLP-1R ligands.

We employed the dual expression system using the BRET-based approach introduced in the previous chapter to investigate the interaction between the GLP-1R and β-arrestins, and we highlight the necessity to profile compounds across multiple signalling pathways and in combination with multiple orthosteric ligands in systems such as the GLP-1R, where more than one endogenous ligand exists.

Results

The addition of Rluc8 to the GLP-1R C-terminus and overexpression of β-Arrestin-venus does not influence GLP-1R pharmacology.

Two stable FlpIN CHO cell lines were generated, one co-expressing cMyc-GLP-1R-Rluc8 and β-Arrestin1-Venus, the other cMyc-GLP-1R-Rluc8 and β-Arrestin2-Venus. To assess the addition of the Rluc8 tag and overexpression of Arrestin-Venus did not affect signalling via the GLP-1R, each cell line was pharmacologically characterised across the three intracellular signalling pathways previously explored in the laboratory. These included cAMP accumulation, iCa^{2+} mobilisation and pERK1/2. We also used whole cell binding to assess ligand affinity. These experiments confirmed that the pharmacology of the cMyc-GLP-1R-Rluc8 expressed in these cells was consistent with the unmodified receptor expressed in the same cell background

without β-arrestin overexpression. Concentration response curves of four GLP-1R peptides and an allosteric ligand (Compound 2) were assessed in each of assays. In agreement with previously published work on the unmodified receptor, these ligands displayed the same rank order in affinity and potency and E_{max} values in cAMP, pERK and iCa^{2+} mobilisation assays when the receptor is tagged with Rluc8 and co-expressed with β-arrestin1-Venus (Figures 5.1 A-D) and β-arrestin2-venus (Figures 5.2 A-D) (exendin-4 > GLP-1(7-36)NH_2 > oxyntomodulin > GLP-1(1-36)NH_2 > Compound 2) . Together, these data indicate that stable expression of the C terminal Rluc8 and the β-arrestin-venus did not significantly influence the pharmacology of the GLP-1R (Table 1.1).

β-Arrestins 1 and 2 are recruited to the GLP-1R when activated by different classes of ligands

To determine ligand-mediated β-arrestin recruitment to the GLP-1R, kinetic experiments were performed over a 20 min time period using the BRET method described in results chapter 4. Five orthosteric peptides (GLP-1(7-36)NH_2, GLP-1(1-36)NH_2, GLP-1(9-36)NH_2, oxyntomodulin and exendin-4, four small molecule ligands (BETP, Compound 2, BOC5 and TT15), and a small peptide (BMS21) (Figure 5.3) were assessed. In addition, two of the small molecules (Compound 2 and BETP) were co-added with each of the peptide agonists to assess their ability to alter the orthosteric ligand-mediated β-arrestin 1 and 2 recruitment (Figures. 5.4-5.7).

Following addition of either 100 nM GLP-1(7-36)NH_2, 100 nM exendin-4 or 1 μM oxyntomodulin, β-arrestin1 was rapidly and transiently recruited to the GLP-1R, peaking at 2.5 min (Figures. 5.4 A-C), however, there was a weak sustained interaction with β-arrestin1 over the timecourse of the experiment (20 min) as the curves do not return back to baseline (vehicle

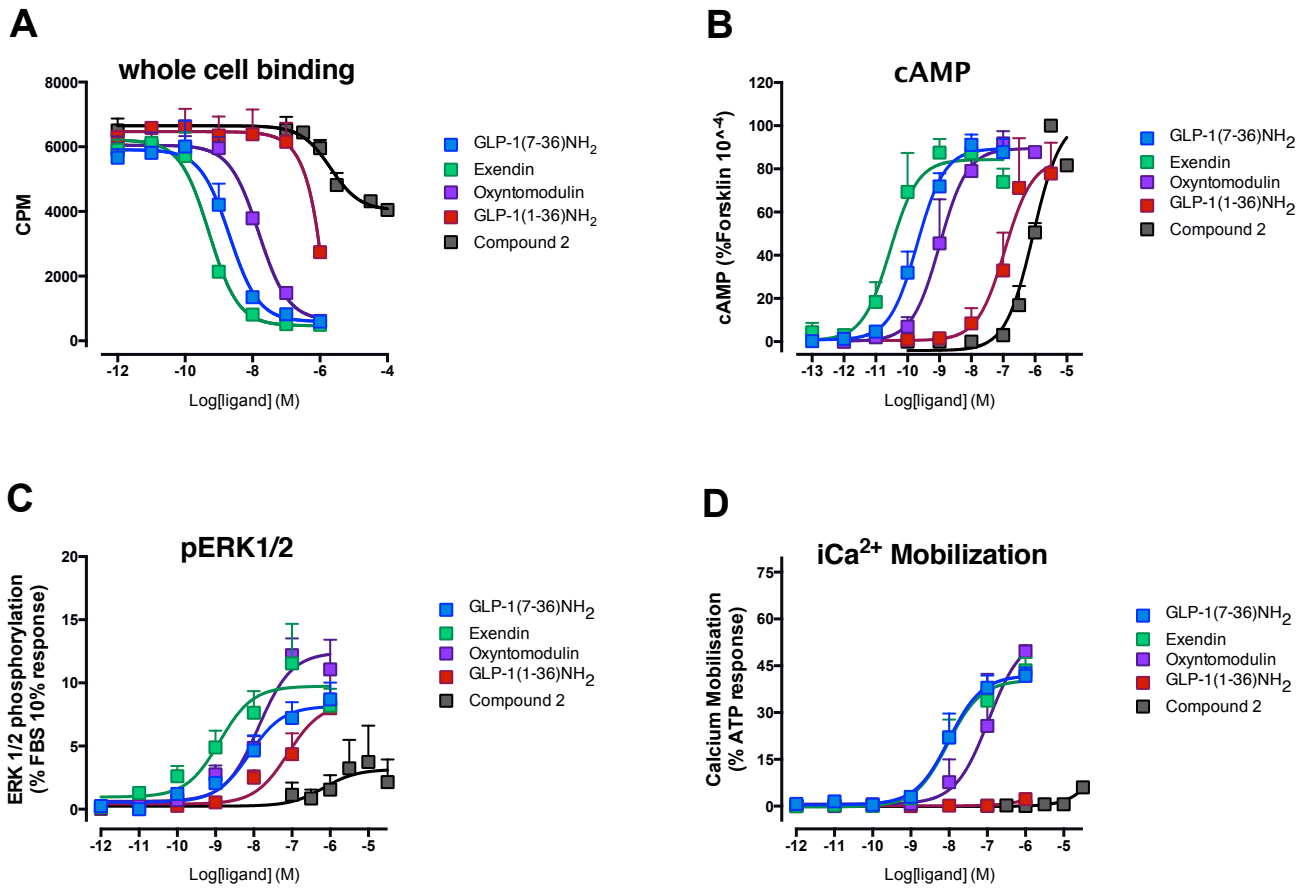

Figure 5.1. Assessment of pharmacology for constructs cMyc-Rluc8-GLP-1R-βArrestin1-Venus in different functional assays.

Whole cell binding (A), cAMP accumulation (B), pERK1/2 (C), iCa^{2+} mobilisation (D), in response to GLP-1R agonists. Concentration response curves were generated in response to GLP-1(7-36)NH_2, exendin, oxyntomodulin, GLP-1(1-36)NH_2 and Compound 2. For cAMP accumulation the E_{max} represents the maximal response normalized to the response elicited by that of 100 nM forskolin; for ERK1/2 phosphorylation the E_{max} represents the maximal response normalised to the response elicited by that of 10 % FBS; for iCa^{2+} mobilization the E_{max} represents the maximal response normalised to the response elicited by that of 10^{-4} M ATP; and analysed using a three-parameter logistic equation. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

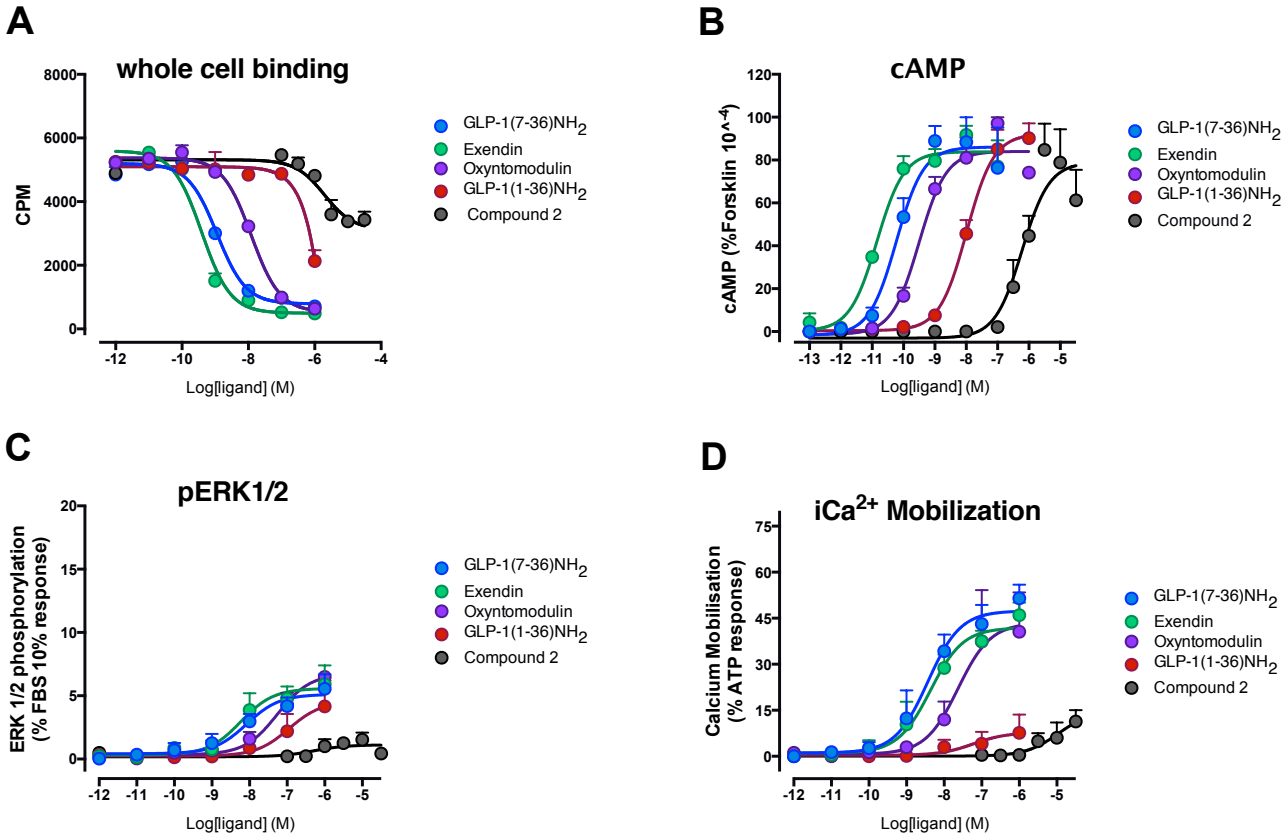

Figure 5.2. Assessment of pharmacology for constructs cMyc-Rluc8-GLP-1R-βArrestin2-Venus in different functional assays.

Whole cell binding (A), cAMP accumulation (B), pERK1/2 (C), iCa^{2+} mobilization (D), in response to GLP-1R agonists. Concentration response curves were generated in response to GLP-1(7-36)NH_2, Exendin, Oxyntomodulin, GLP-1(1-36)NH_2 and Compound 2. For cAMP accumulation the E_{max} represents the maximal response normalised to the response elicited by that of 100 nM forskolin; for ERK1/2 phosphorylation the E_{max} represents the maximal response normalised to the response elicited by that of 10 % FBS; for iCa^{2+} mobilization the E_{max} represents the maximal response normalised to the response elicited by that of 10^{-4} M ATP; and analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Table 5.1. Comparison of the pharmacology of different cMyc tagged GLP-1R constructs in CHOFlpIn cells. Data were analyzed using a three-parameter logistic equation as defined in eq. 1. pIC_{50} values represent the negative logarithm of the concentration of ligand that inhibits 50 % of the binding of the radiolabelled antagonist ^{125}I-Exendin(9-39). pEC_{50} values represent the negative logarithm of the concentration of agonist that produces half the maximal response. The E_{max} represents the maximal response normalized to the response elicited by that of 100 nM forskolin, 10% FBS or 100 μM ATP for cAMP accumulation, pERK1/2 and iCa^{2+} mobilization respectively; and for β-Arrestin1 and β-Arrestin2, the response of each ligand in mBRET units. These values were derived from concentration response curves in Figures 5.1, 5.2, 5.9 and 5.10; this information was used to derive bias factors. All values are mean ± S.E.M. of four to five independent experiments, conducted in duplicate (ND = no detectable response).

		cMyc tagged GLP-1R (CHO)			cMyc-GLP-1R-Rluc8-βArrestin1-Venus			cMyc-GLP-1R-Rluc8-βArrestin2-Venus		
	Ligand	**pIC_{50}**	**pEC_{50}**	**E_{max}**	**pIC_{50}**	**pEC_{50}**	**E_{max}**	**pIC_{50}**	**pEC_{50}**	**Emax**
Whole Cell Binding	**GLP-1(7-36)NH_2**	9.2±0.1	-	-	8.7±0.1	-	-	8.9±0.1	-	-
	Exendin	9.6±0.1	-	-	9.3±0.1	-	-	9.4±0.1	-	-
	Oxyntomodulin	7.6±0.2	-	-	7.8±0.1	-	-	7.9±0.1	-	-
	GLP-1(1-36)NH_2	6.4±0.2	-	-	ND	-	-	ND	-	-
	Compound 2	6.1±0.4	-	-	5.7±0.2	-	-	5.7±0.2	-	-
	BETP	ND			ND			ND		
cAMP	**GLP-1(7-36)NH_2**	-	10.4 ± 0.1	92 ± 2	-	9.7 ± 0.1	89± 3	-	10.2 ±0.2	86 ± 7
	Exendin	-	10.9 ± 0.1	90 ± 3	-	10.6 ± 0.1	84 ± 5	-	10.9 ± 0.1	84 ± 4
	Oxyntomodulin	-	9.0 ± 0.1	89 ± 6	-	9.0 ±0.2	89 ± 5	-	9.5 ±0.1	84 ± 4
	GLP-1(1-36)NH_2	-	8.1 ± 0.1	91 ± 7	-	7.60 ± 0.2	84 ± 8	-	7.9 ± 0.1	92 ± 4
	Compound 2	-	5.8 ± 0.1	79 ± 6	-	6.1 ± 0.2	83 ± 10	-	6.2 ± 0.2	79 ± 8
	BETP		5.2 ± 0.1	14 ± 5						
pERK1/2	**GLP-1(7-36)NH_2**	-	7.9 ± 0.1	10.1 ± 0.3	-	8.1 ± 0.2	8.2 ± 0.7	-	8.1 ± 0.3	5.1 ± 0.5
	Exendin	-	8.4 ± 0.1	**11** ± 0.7	-	8.9 ± 0.3	9.7 ± 1.0	-	8.2 ± 0.3	5.6 ± 0.6
	Oxyntomodulin	-	7.4 ± 0.1	12 ± 1.1	-	7.3 ± 0.2	7.8 ± 0.2	-	7.3 ± 0.2	6.7 ± 0.5
	GLP-1(1-36)NH_2	-	7.1 ± 0.1	11 ± 1.2	-	7.0 ± 0.5	7.1 ± 0.4	-	7.0 ± 0.5	4.6 ± 0.5
	Compound 2	-	6.4 ± 0.1	3 ± 0.5	-	6.2 ± 1	3.2 ± 1.2	-	6.2 ± 0.8	1.7 ± 0.1
	BETP		ND	ND						
Ca^{2+}	**GLP-1(7-36)NH_2**	-	8.1 ± 0.1	42 ± 4	-	8.0 ± 0.2	42 ± 3	-	7.8 ± 0.2	25.8 ± 1.5
	Exendin	-	7.7 ± 0.1	**35**± 2	-	8.0 ± 0.1	40 ± 2	-	8.0 ± 0.3	23.1 ± 2.5
	Oxyntomodulin	-	6.5 ± 0.7	35 ± 6	-	7.0 ± 0.3	26 ± 5	-	7.6 ± 0.4	25.9 ± 5.5
	GLP-1(1-36)NH_2	-	ND	ND	-	ND	ND	-	ND	ND
	Compound 2	-	ND	ND	-	ND	ND	-	ND	ND
	BETP	-	5.0 ± 0.3	13 ± 6					5.0 ± 0.5	14.8 ± 6.5

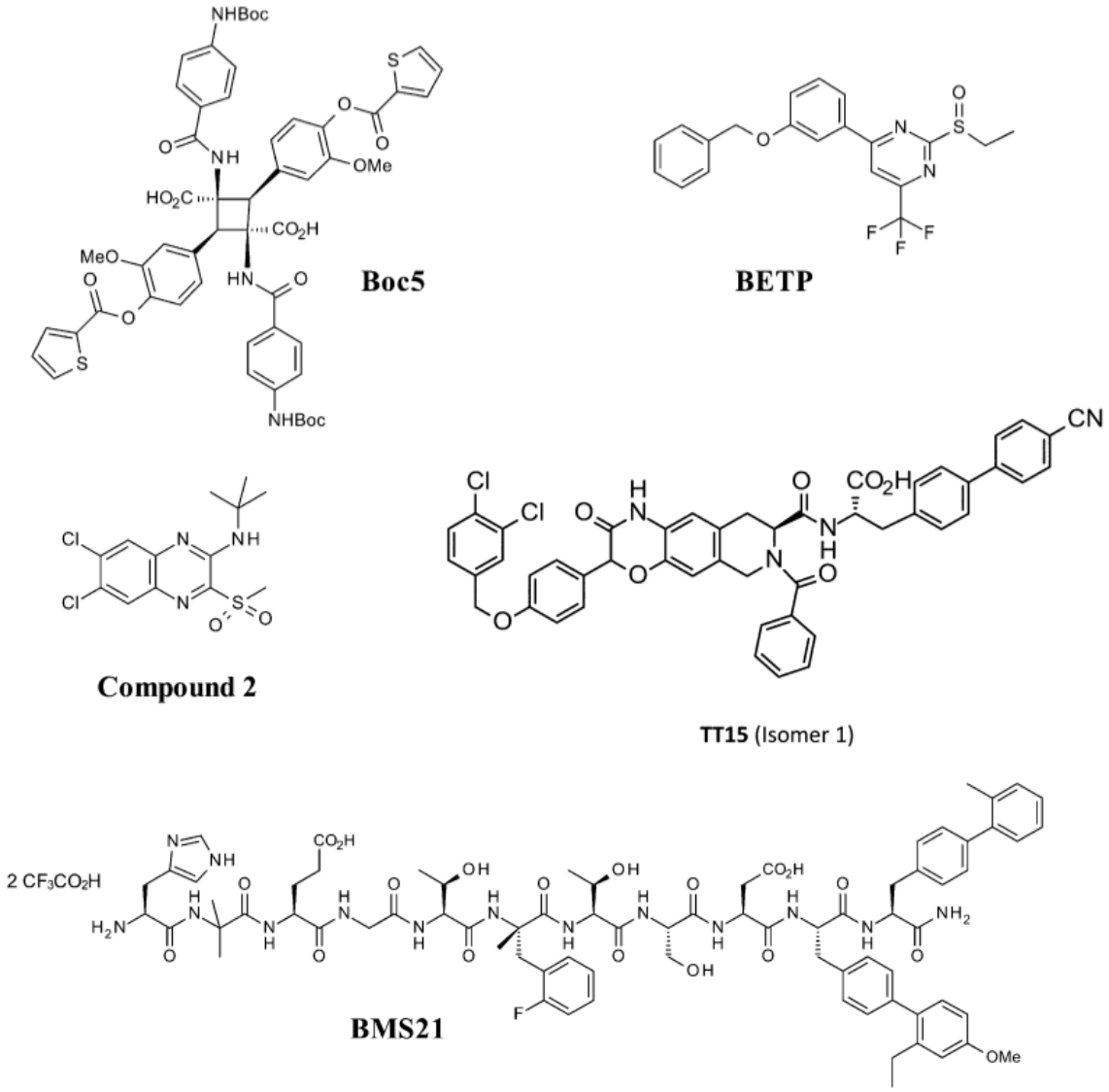

Figure 5.3. Small molecule compounds of the GLP-1R A) Boc5, B) BETP, C) Compound 2 D) TT15, E) BMS21

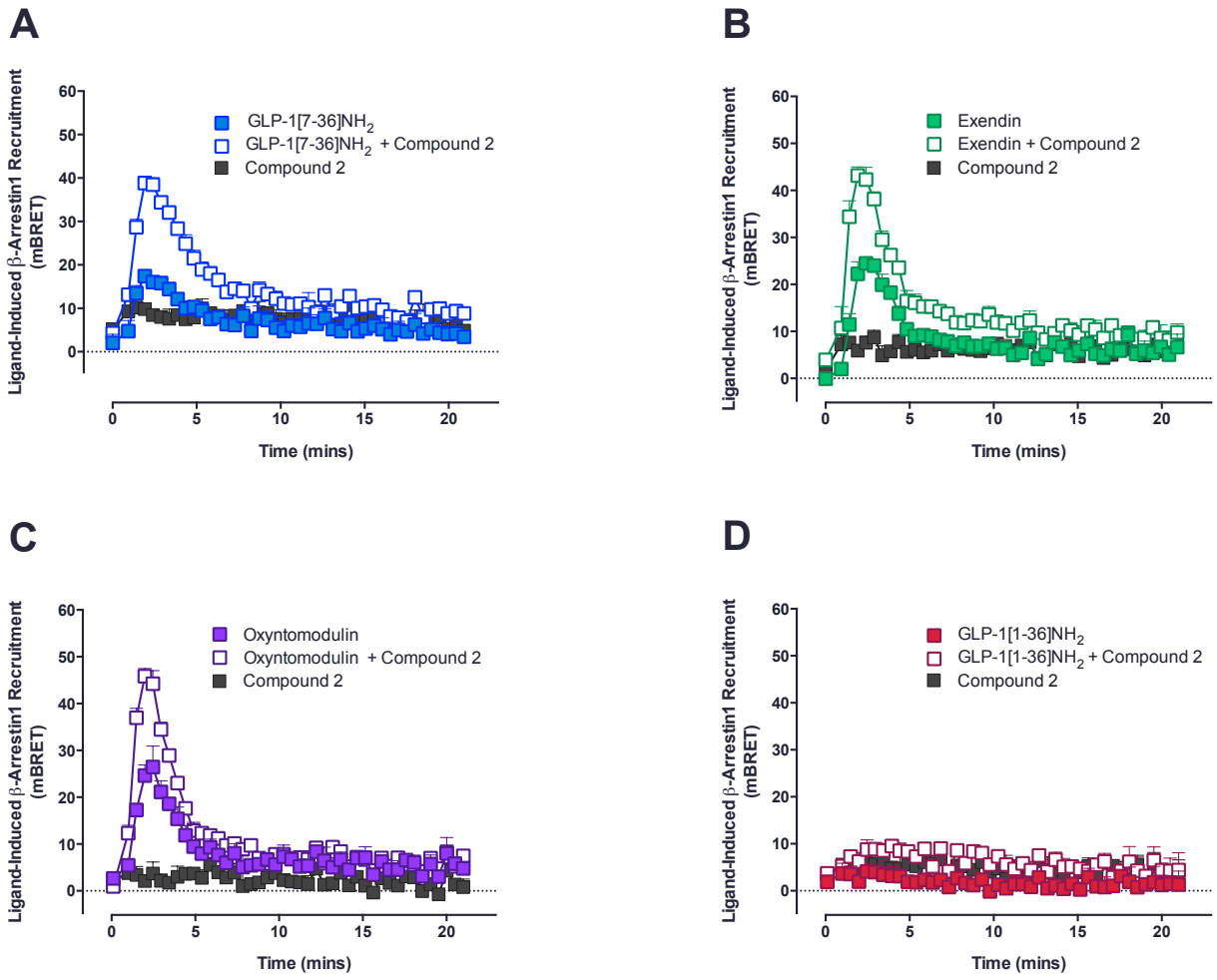

Figure 5.4 β-Arrestin1 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator Compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

control). In contrast, GLP-1(1-36)NH_2 and GLP-1(9-36)NH_2 were unable to recruit detectable levels of β-arrestin1 at the concentration tested (30 μM) (Figure 5.4 D; 5.14 A, B).

Stimulation of the GLP-1R with 1 μM Compound 2 induced weak recruitment of β-arrestin1 with a peak response at approximately 3 min that remained sustained throughout the timecourse (Figure 5.4). Interestingly, co-addition of 1 μM Compound 2 with each of the peptides increased the transient recruitment of β-arrestin1 mediated by the peptide. For GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin, the recruitment was enhanced by 56 %, 44 % and 43 % respectively (Figures. 5.4 A-C). However, co-addition of Compound 2 with GLP-1(1-36)NH_2 displayed the same profile as Compound 2 alone, suggesting that there was no modulation of the peptide response by the allosteric ligand (Figure 5.4 D).

In addition to Compound 2, the allosteric ligand, BETP, also recruited β-arrestin1 to the GLP-1R, peaking at 3 min post addition. While still a partial agonist compared to the peptide ligands, this small molecule induced a stronger response than Compound 2, but with the same profile (a sustained recruitment). However, unlike Compound 2, BETP only weakly increased GLP-1(7-36)NH_2 and Oxyntomodulin-mediated recruitment of β-arrestin1 (Figures. 5.5 A and C), whereas there was little notable difference observed for exendin-4 (Figure 5.5 B). Similar to Compound 2, co-addition of BETP and GLP-1(1-36)NH_2 showed no distinguishable β-arrestin1 recruitment over that of BETP alone (Figure 5.5 D).

The synthetic compounds Boc5 and TT15, and the small 11 mer peptide BMS21 were unable to recruit β-Arrestin1 to the receptor at the concentrations tested (10 μM, 10 μM and 1μM respectively) (Figure 5.6 A).

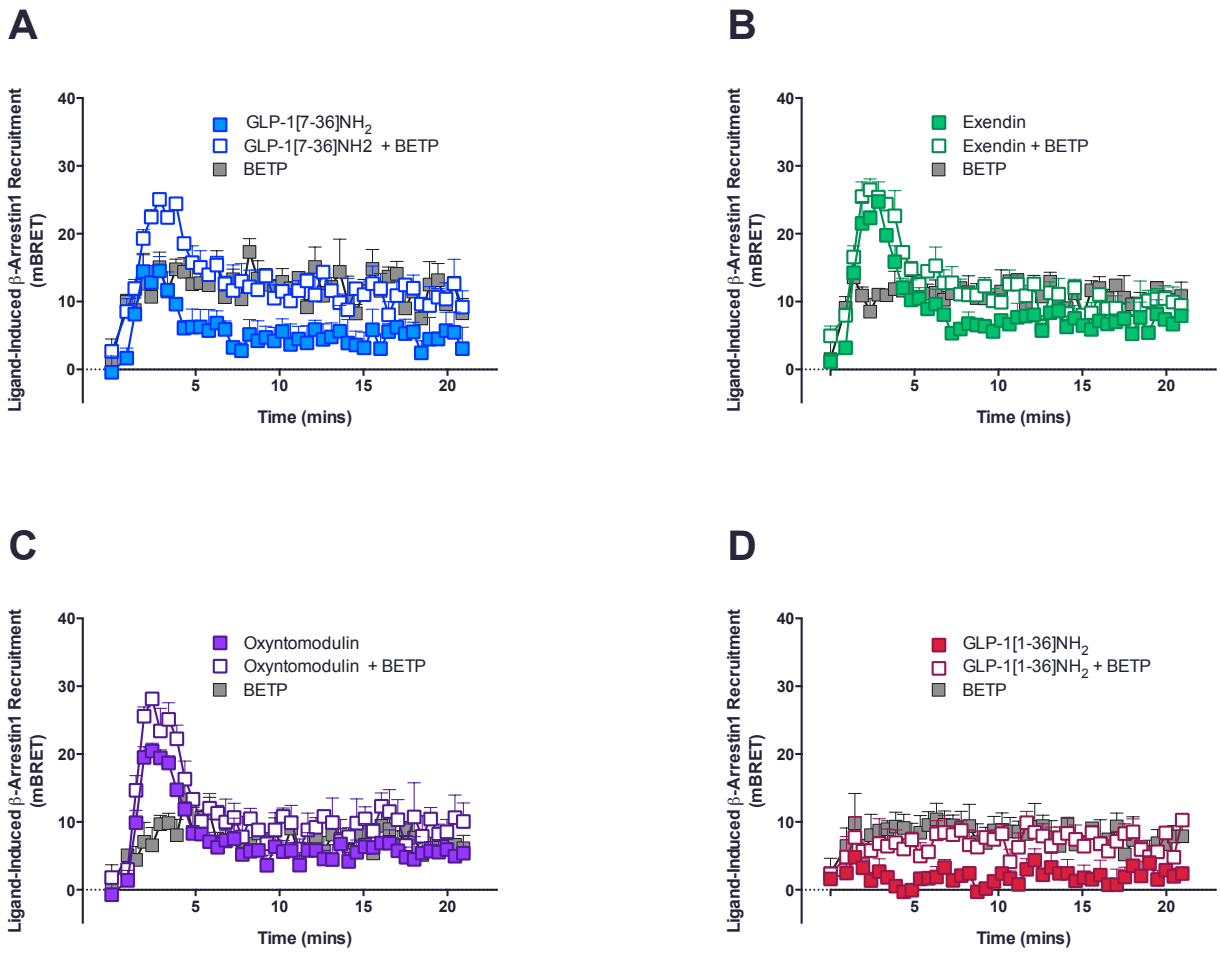

Figure 5.5. β-Arrestin1 recruitment time courses of GLP-1R agonists.

$GLP\text{-}1(7\text{-}36)NH_2$ (A), Exendin (B), Oxyntomodulin (C), and $GLP\text{-}1(1\text{-}36)NH_2$ (D), mediated β-arrestin1 recruitment, in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

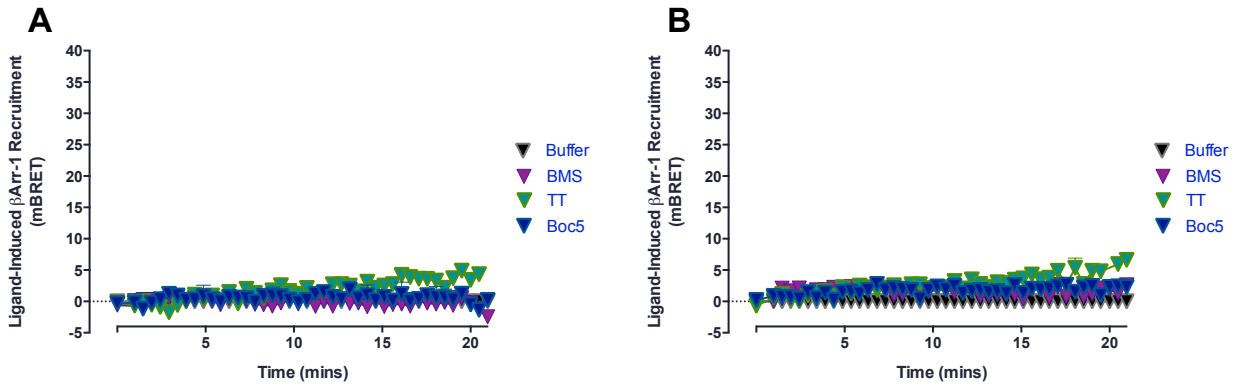

Figure 5.6. BMS21, TT and Boc5 mediated recruitment of β-arrestin1 and β-arrestin2 to the GLP-1R.

Kinetic profiles were generated to asses BMS21, TT and Boc5 mediated recruitment of both β-arrestin1 (A) and β-arrestin2 (B) at submaximal concentrations. All values are mean ± S.E.M. of three independent experiments performed in duplicate.

Similar to β-arrestin1, β-arrestin2 was also rapidly and transiently recruited to the GLP-1R within 2.5 min post addition of 100 nM GLP-1(7-36)NH_2, exendin-4 or 1 μM oxyntomodulin (Figure 5.7A-C) and there was no detectable recruitment with 1 μM of GLP-1(1-36)NH_2. In addition, a sustained interaction between the GLP-1R and arrestin 2 was observed throughout the timecourse. Compound 2 induced only weak recruitment that was also sustained throughout the timecourse of the experiment (Figure 5.7 D). Co-addition of 1 μM Compound 2 with GLP-1(7-36)NH_2, exendin-4 or oxyntomodulin elevated the response by 46%, 31% and 34% above peptide alone, respectively (Figures. 5.7 A-C). There was no change from Compound 2 response alone with co-addition of GLP-1(1-36)NH_2 and Compound 2 on the β-arrestin2 recruitment profile.

BETP addition alone could also recruit β-arrestin2 in a sustained manner, but this was weaker than that observed for β-arrestin1. In the presence of BETP, GLP-1(7-36)NH_2 and oxyntomodulin-mediated β-arrestin2 recruitment was 51 % and 21 % above peptide response alone respectively (Figures. 5.8 A and C). Consistent with that observed for β-arrestin1 recruitment, BETP had little effect on exendin-4 or GLP-1(1-36)NH_2 –mediated β-arrestin2 recruitment at the concentrations of ligands assessed in these experiments (Figures 5.8 B and D). As observed for β-arrestin1, Boc5, TT15 and BMS21 were unable to recruit β-arrestin2 to the GLP-1R at the concentrations tested (10 μM, 10 μM and 1μM respectively) (Figure 5.6 B).

These data provide information on the peak time in which peptides, compound or co-addition recruit β-arrestins to the GLP-1R. This peak time was used to generate concentration response curves in the following section. As Boc5, TT15 and BMS21 did not recruit either β-arrestin1 or β-arrestin2, these compounds were not assessed further.

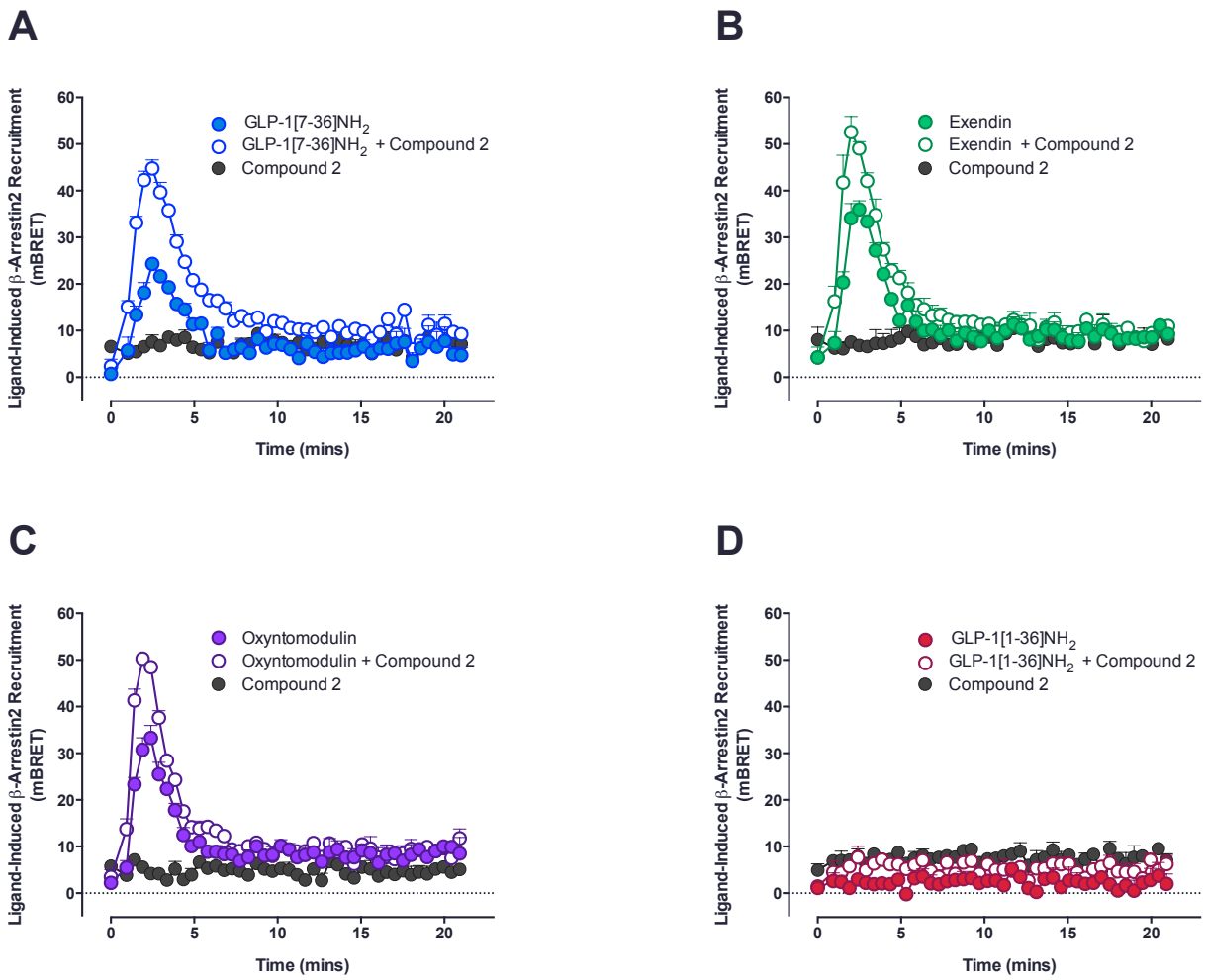

Figure 5.7. β-Arrestin2 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36)NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator Compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

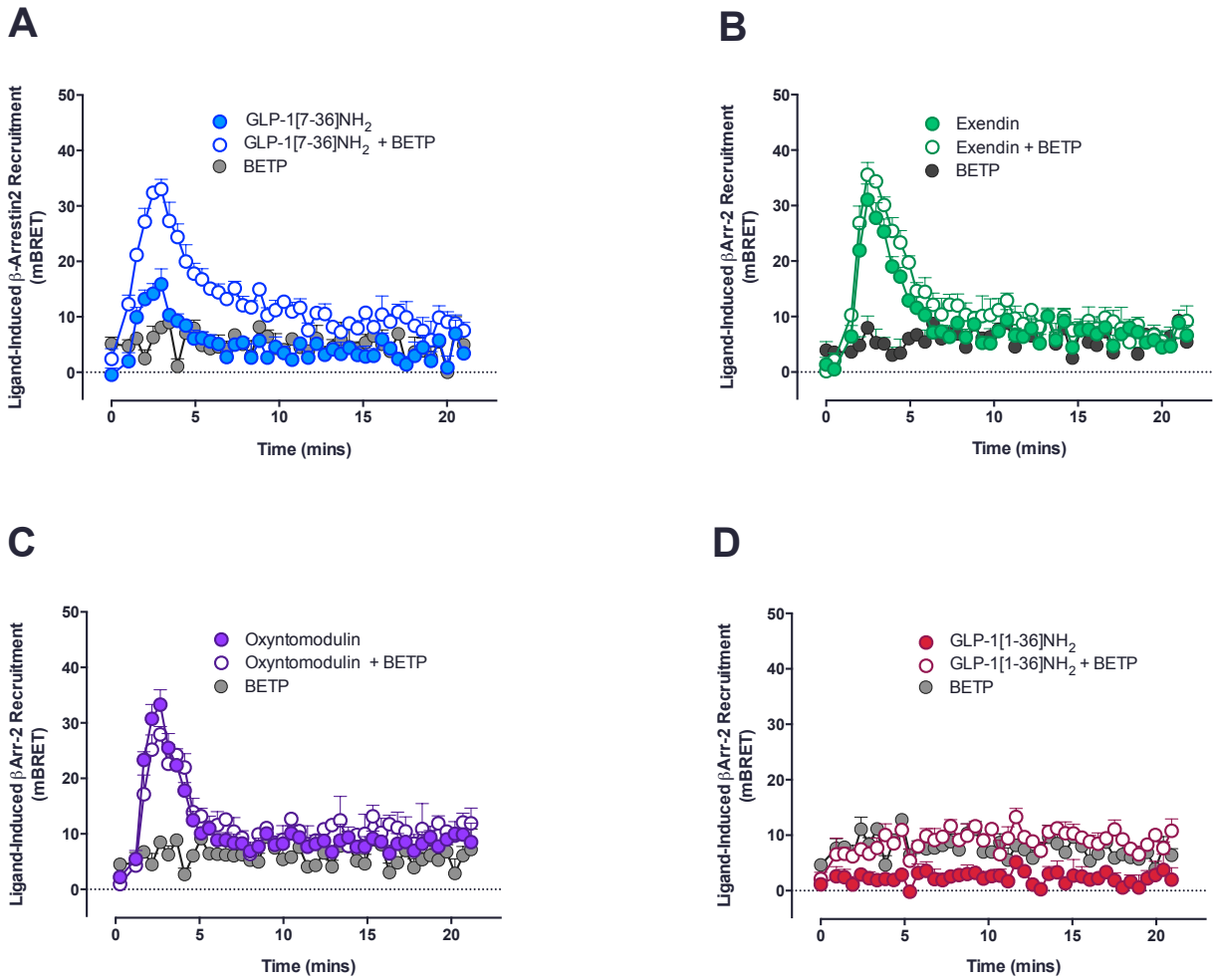

Figure 5.8. β-Arrestin2 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36)NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Orthosteric peptides and allosteric small molecule ligands recruit β-arrestins to the GLP-1R in a concentration dependent manner.

Concentration response curves for peptide-mediated β-arrestin1 and β-arrestin2 recruitment were generated at the peak response time point determined above for each peptide (Figures. 5.9 A,B Table 2), Compound 2 and BETP (Figures. 5.10 A,B Table 5.2). In accordance with the timecourse experiments, GLP-1(7-36)NH_2, exendin-4, and oxyntomodulin recruited both β-arrestins. In line with their affinities, exendin-4 had the highest potency, followed by GLP-1(7-36)NH_2 and oxyntomodulin with pEC_{50} values of 8.4 ± 0.1, 7.7 ± 0.1 and 6.8 ± 0.1 respectively, (Figures. 5.9 A, B Table 1). As expected, no β-arrestin recruitment could be detected in the presence of GLP-1(1-36)NH_2 (Figure 5.9, Table 5.2). Both BETP and Compound 2 were weak equipotent partial agonists for β-arrestin1 and β-arrestin2 recruitment (Figures. 5.10 A, B, Tables 5.2, 5.3).

Compound 2 and BETP selectively modulate peptide-mediated β-arrestin recruitment to the GLP-1R

Concentration response curves were generated for each peptide ligand in the absence and presence of increasing concentrations of either Compound 2 or BETP (Figures. 5.11- 5.14). Analysis of these interaction curves with the operational model of allosterism (Equation 4 of Chapter 2 materials and methods) was performed to derive a *p*Kb (affinity) of the allosteric ligand and a cooperativity value (αβ) that describes the allosteric effect between the peptide and the small molecule. This revealed that both Compound 2 and BETP differentially modulated β-arrestin recruitment in a ligand-dependent manner (Table 5.4). Compound 2 positively modulated all three peptides in both β-arrestin1 and β-arrestin2 pathways (Figures. 5.11-5.12; Table 5.4), however to different extents. β-arrestin1 recruitment by GLP-1(7-36)NH_2 and

oxyntomodulin was modulated by 12-fold and 11-fold, respectively, with similar effects observed for β-arrestin2 (11-

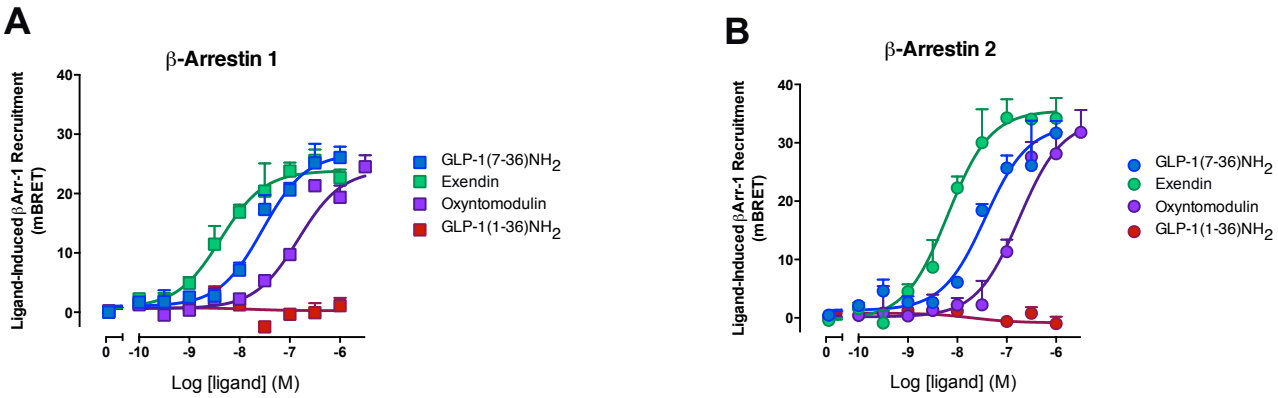

Figure 5.9 Peptide-induced recruitment of β-Arrestins to the GLP-1R. Concentration response curves for β-arrestin1 (A) and β-arrestin2 (B) recruitment to the GLP-1R for GLP-1(7-36)NH_2, Exendin, Oxyntomodulin and GLP-1(1-36)NH_2. Data are normalised to the response elicited by GLP-1(7-36)NH_2 and analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

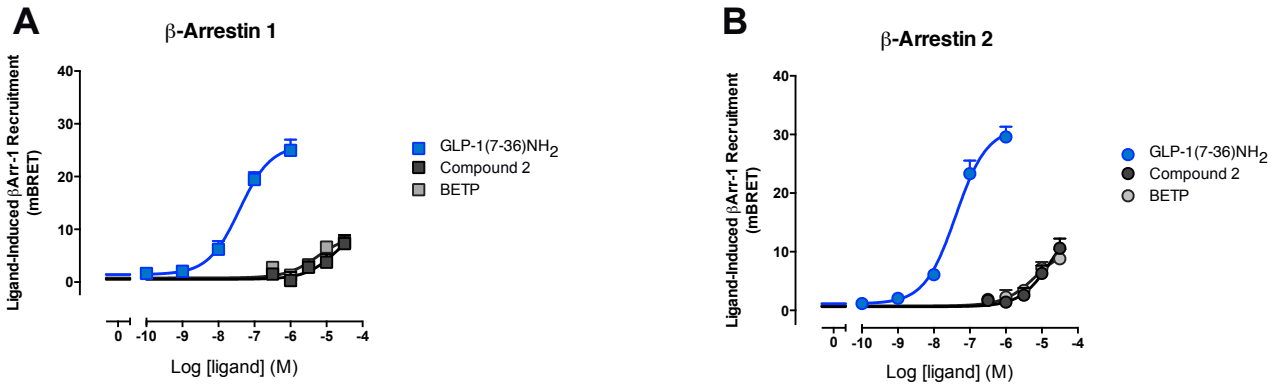

Figure 5.10 Small molecule-induced recruitment of β-Arrestins to the GLP-1R. Concentration response curves for β-arrestin1 (A) and β-arrestin2 (B) recruitment to the GLP-1R for GLP-1(7-36)NH_2, Compound 2 and BETP. Data are normalized to the response elicited by GLP-1(7-36)NH_2 and analysed using a three-parameter logistic equation. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Table 5.2

Differential effects of peptide agonists of the human GLP-1R in β-arrestin1 and β-arrestin2 recruitment in FlpInCHO cells stably expressing the human GLP-1R. pKa values are the functional affinity values of the peptides/small molecule derived from an operational model. pEC_{50} values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. All values are mean ± S.E.M. of three to five independent experiments, conducted in duplicate (ND = no detectable response).

	βArrestin 1			**βArrestin 2**		
Ligand	pKa	pEC_{50}	E_{max}	pKa	pEC_{50}	E_{max}
GLP-1(7-36)NH_2	8.7 ± 0.1	7.7 ± 0.1	100 ± 6	8.9 ± 0.1	7.4 ± 0.1	100 ± 5
Exendin	9.3 ± 0.1	8.4 ± 0.1	89 ± 3	9.4 ± 0.1	8.2 ± 0.1	108 ± 5
Oxyntomodulin	7.8 ± 0.1	6.8 ± 0.1	90 ± 4	7.9 ± 0.1	6.8 ± 0.1	102 ± 7
GLP-1(1-36)NH_2	-	ND	ND	-	ND	ND

Table 5.3

Differential effects of peptide/small molecule agonists of the human GLP-1R in β-arrestin1 and β-arrestin2 recruitment in FlpInCHO cells stably expressing the human GLP-1R. pKa values are the functional affinity values of the peptides/small molecule derived from an operational model. pEC_{50} values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. All values are mean ± S.E.M. of three to five independent experiments, conducted in duplicate.

	βArrestin 1			**βArrestin 2**		
Ligand	pKa	pEC_{50}	E_{max}	pKa	pEC_{50}	E_{max}
GLP-1(7-36)NH_2	8.7 ± 0.1	7.6 ± 0.1	100 ± 6	8.9 ± 0.1	7.4 ± 0.1	100 ± 5
Compound 2	5.7 ± 0.2	5.0 ± 0.2	30 ± 5	5.7 ± 0.2	4.8 ± 0.2	51 ± 0
BETP	-	5.0 ± 0.2	40 ± 7	-	5.0 ± 0.1	63 ± 15

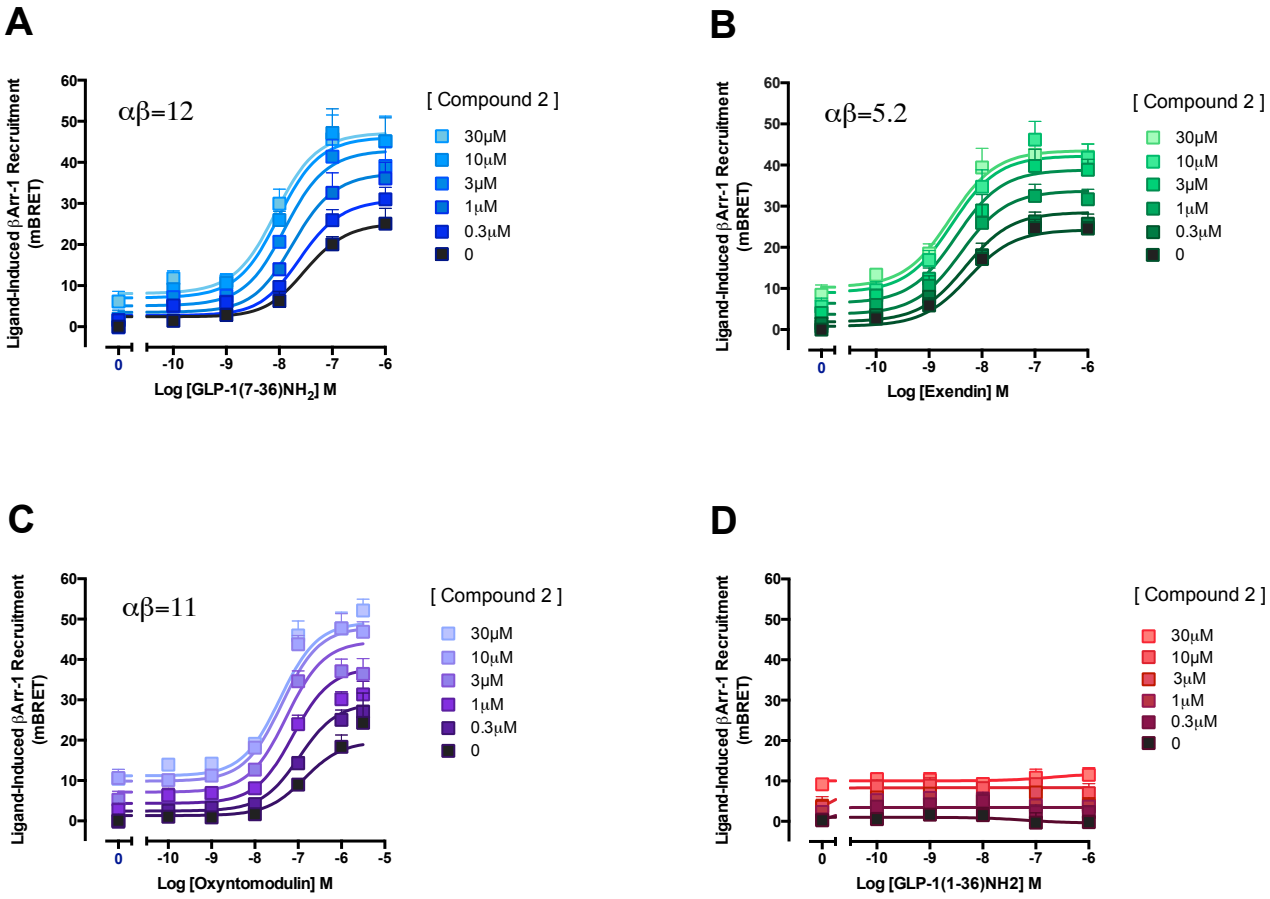

Figure 5.11. Compound 2 potentiates peptide mediated recruitment of β-arrestin1 to the GLP-1R.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of Compound 2. Compound 2 did not potentiate GLP-1R-mediated recruitment of β-arrestin1 by GLP-1(1-36)NH_2. The curves represent the best global fit of an operational model of allosterism (eq. 5). Shown for each graph is the cooperativity factor (αβ), that describes the interaction between the peptide and the allosteric ligand. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

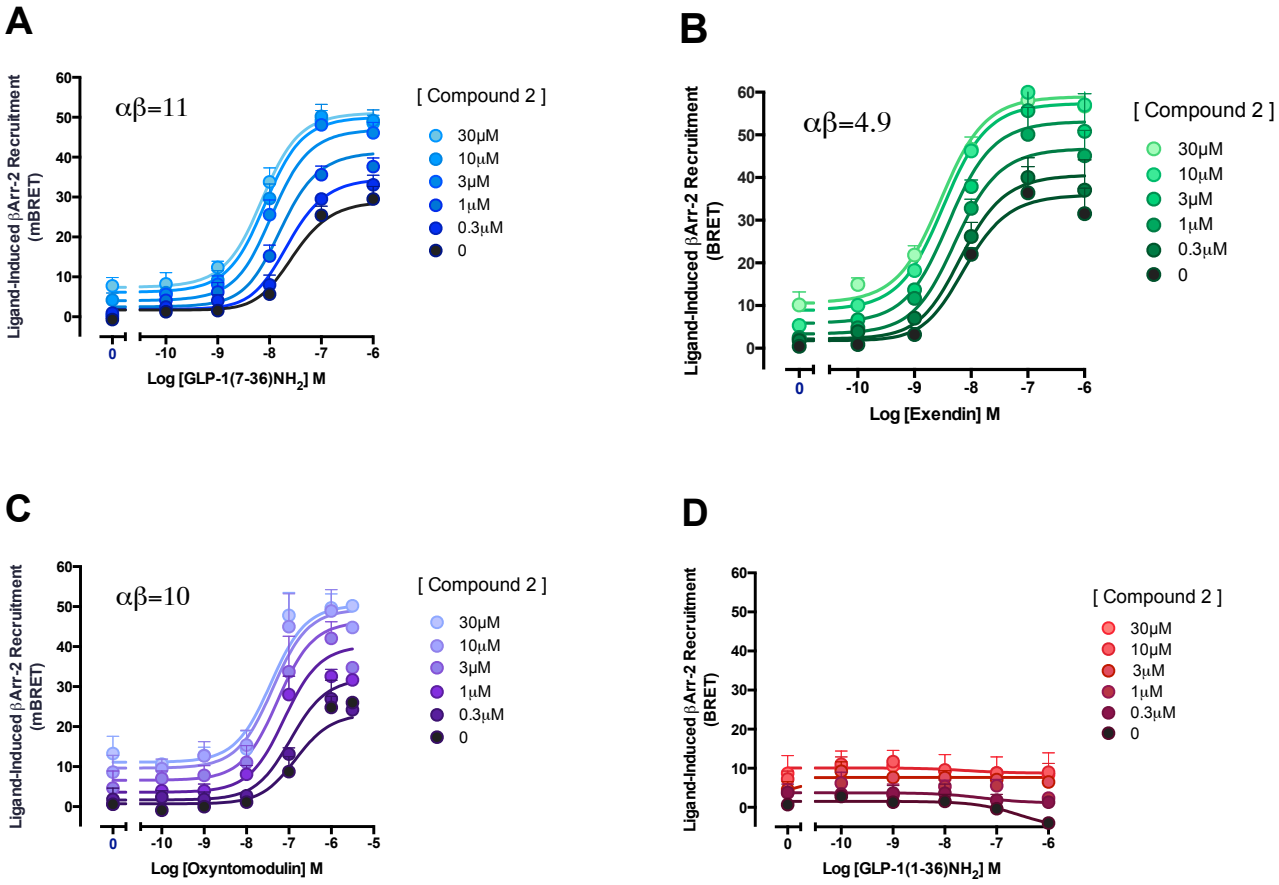

Figure 5.12. Compound 2 potentiates peptide mediated recruitment of β-arrestin2 to the GLP-1R.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of Compound 2 for β-Arrestin2 recruitment. Compound 2 did not potentiate GLP-1R-mediated recruitment of β-arrestin2 by GLP-1(1-36)NH_2. The curves represent the best global fit of an operational model of allosterism (eq. 5). Shown for each graph is the cooperativity factor (αβ), that describes the interaction between the peptide and the allosteric ligand. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate

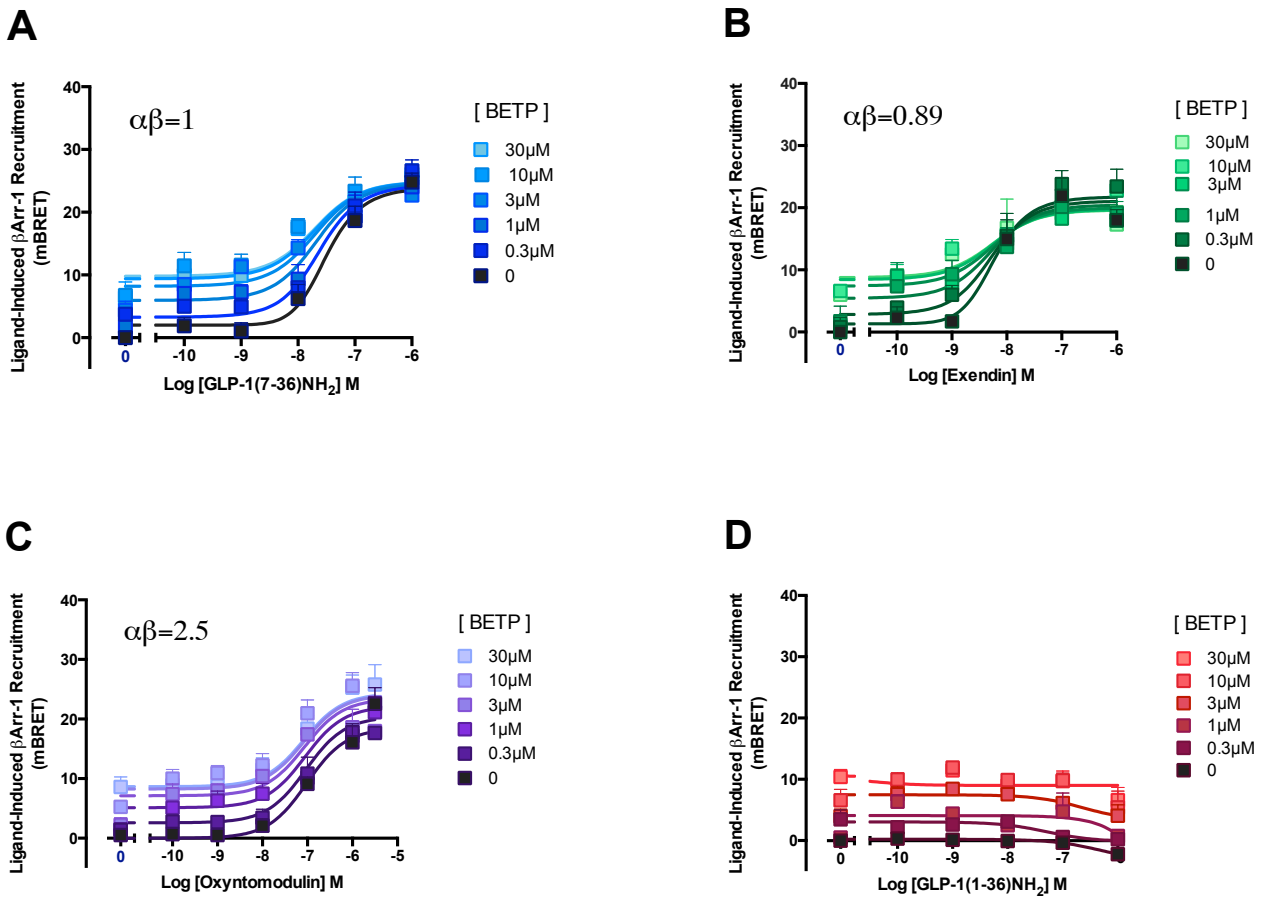

Figure 5.13. BETP does not significantly alter peptide mediated recruitment of β-arrestin1 to the GLP-1R.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of BETP for β-arrestin1 recruitment. BETP did not potentiate GLP-1R-mediated recruitment of β-arrestin by GLP-1(1-36)NH_2. The curves represent the best global fit of an operational model of allosterism (eq. 5). Shown for each graph is the cooperativity factor (αβ), that describes the interaction between the peptide and the allosteric ligand. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

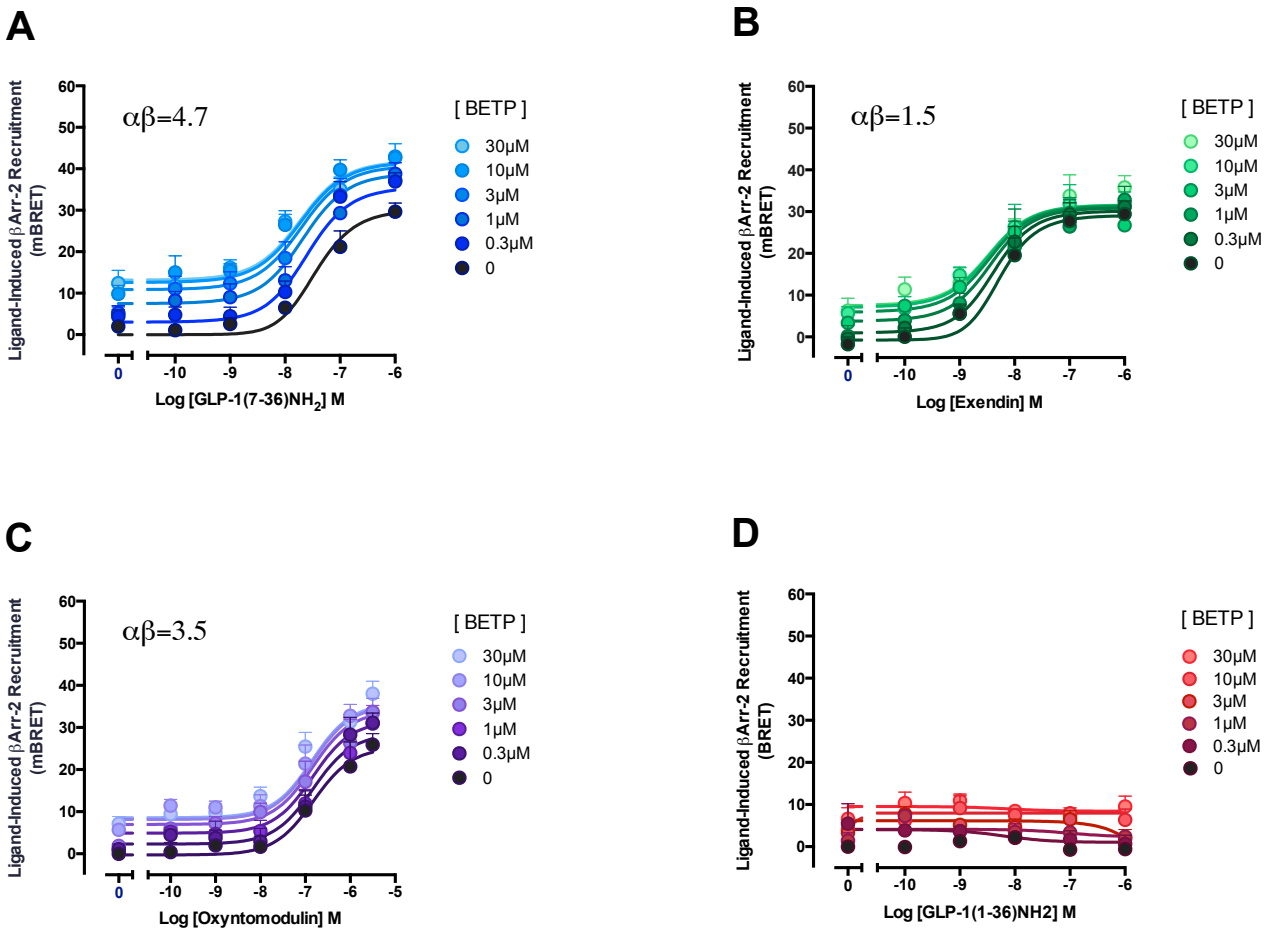

Figure 5.14. BETP does not significantly alter peptide mediated recruitment of β-arrestin2 to the GLP-1R. .

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of BETP for β-Arrestin2 recruitment. BETP did not potentiate GLP-1R-mediated recruitment of β-arrestin2 by GLP-1(1-36)NH_2. The curves represent the best global fit of an operational model of allosterism (eq. 5). Shown for each graph is the cooperativity factor (αβ), that describes the interaction between the peptide and the allosteric ligand. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

Table 5.4. Functional cooperativity estimates for the interaction between BETP, or Compound 2, and GLP-1R peptide ligands for β arrestin 1 and 2 pathways.

Data were derived from analysis of interaction dose-response curves with an operational model of allosterism as defined in equation 4. pKb values are the negative logarithms for the functional affinity of the allosteric ligands, log αβ represents the composite affinity/efficacy cooperativity between the allosteric ligand and the orthosteric peptide ligand. Antilogarithms are shown in parentheses. Values represent the mean ± S.E.M of four to six independent experiments performed in duplicate. Data were analysed with one-way analysis of variance and Dunnett's post test (* $p < 0.05$) (NR = no response).

Pathway	Allosteric Ligand	pKb	Log αβ (αβ)			
			Exendin-4	GLP-1(7-36)NH_2	Oxyntomodulin	GLP-1(1-36)NH_2
β-Arr1	Cpd2	5.27 ± 0.18	0.72 ± 0.18 (5.2)*	1.07 ± 0.19 (12)*	1.05 ± 0.14 (11)*	NR
	BETP	5.42 ± 0.17	-0.05 ± 0.04 (0.89)	-0.01 ± 0.02 (1.0)	0.40 ± 0.17 (2.5)	NR
β-Arr2	Cpd2	5.30 ± 0.19	0.69 ± 0.20 (4.9)*	1.06 ± 0.13 (11)*	0.99 ± 0.19 (10)*	NR
	BETP	5.38 ± 0.16	0.18 ± 0.26 (1.5)	0.67 ± 0.18 (4.7)	0.54 ± 0.19 (3.5)	NR
cAMP	Cpd2	5.43 ± 0.29	0.24 ± 0.3 (1.7)	0.22± 0.28 (1.7)	1.48 ± 0.27 (29)*	0.31 ± 0.17 (2.0)
	BETP	5.01 ± 0.04	0.45 ± 0.20 (2.8)	0.31 ± 0.18 (2.0)	1.21 ± 0.17 (16)*	0.20 ± 0.12 (1.6)
ERK1/2	Cpd2	5.29 ± 0.19	-0.77 ± 0.21 (5.9)	-0.48 ± 0.17(0.33)	-0.21 ± 0.13 (0.62)	-0.44 ± 0.20 (0.36)
	BETP	5.46 ± 0.29	-0.9 ± 0.21 (0.13)*	-1.03 ± 0.23 (0.09)*	-0.44 ± 0.19 (0.36)	-1.85 ± 0.88 (0.01)
iCa^{2+}	Cpd2	5.58 ± 0.38	0.28 ± 0.15 (1.9)	-0.20 ± 0.15 (0.63)	0.14 ± 0.16 (1.4)	NR
	BETP	4.83 ± 0.16	1.0 ± 0.26 (10)*	0.58 ± 0.19 (3.8)	0.23 ± 0.11 (1.7)	NR

fold and 10-fold, respectively). While exendin-4-mediated recruitment of both β-arrestin1 and β-arrestin2 was also enhanced by Compound 2, this was to a lesser extent (5-fold). In contrast, BETP did not significantly modulate any peptide for recruitment of either β-arrestin1 or β-arrestin2. However, despite not reaching significance, there was weak modulation by BETP of GLP-1(7-36)NH_2 in β-arrestin2 (but not β-arrestin1) and weak modulation of oxyntomodulin (2 - 4 fold) for both effectors (Figures. 5.13-5.14; Table 5.4).

Compound 2 and BETP selectively modulate the ability of the metabolite, GLP-1(9-36)NH_2-to recruit β-arrestin1 to the GLP-1R

We investigated the potential of the metabolite GLP-1(9-36)NH_2 to recruit β-arrestin1 and also the ability of the allosteric ligands to modulate this activity at the GLP-1R. Although the metabolite was unable to promote recruitment of β-arrestin1, both small allosteric molecules Compound 2 and BETP were able to induce GLP-1(9-36)NH_2-mediated recruitment of β-arrestin1. Increasing concentrations of either Compound 2 or BETP displayed modest potentiation of this response, however a cooperativity factor could not be derived as the data did not fit the operational model of allosterism (Fig. 15A-B).

GLP-1R ligands display significant signalling bias in coupling to cellular effectors.

The GLP-1R couples to multiple intracellular signalling components, and thus has the ability to display stimulus bias towards particular pathways. These effects on ligand bias can be readily observed in bias plots, which display the response observed at equimolar concentrations of ligand for one pathway relative to another (Figure 5.16).

In this visual representation, all GLP-1R ligands studied appear to display no apparent bias between β-arrestin1 and β-arrestin2 recruitment as all ligands lie along the line of identity (LOI)

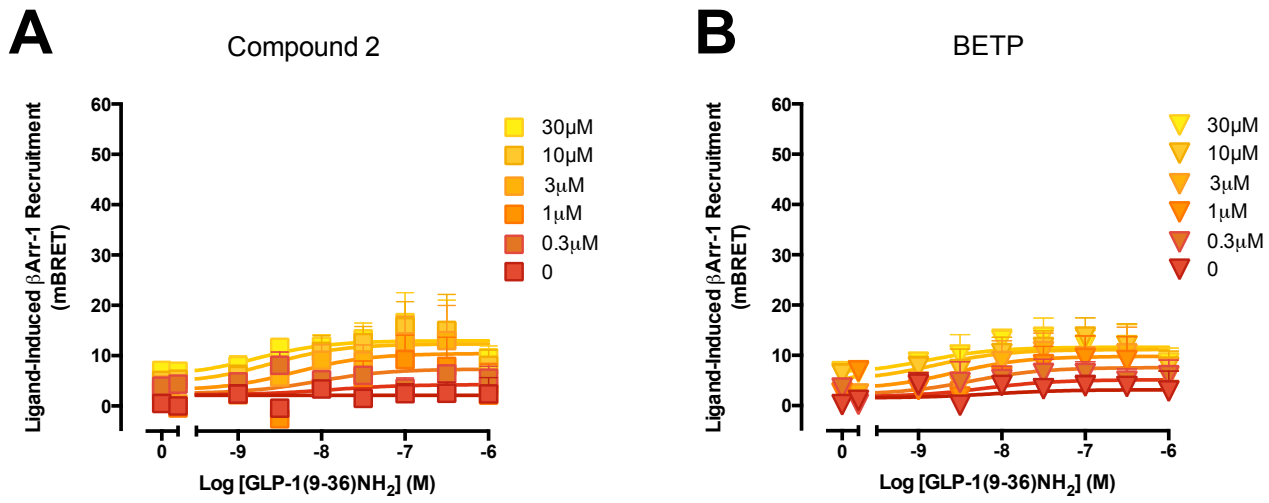

Figure 5.15. Compound 2 and BETP weakly potentiate GLP-1R-mediated recruitment of β-arrestin1 by the GLP-1 metabolite GLP-1(9-36)NH2.
Concentration response curves were generated for GLP-1(9–36)NH_2 in the absence and presence of increasing concentrations of Compound 2 (A) and BETP (B) for β-arrestin1 recruitment. Compound 2 and BETP only modestly potentiated GLP-1R-mediated recruitment of β-arrestin1 by GLP-1(9-36)NH_2. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

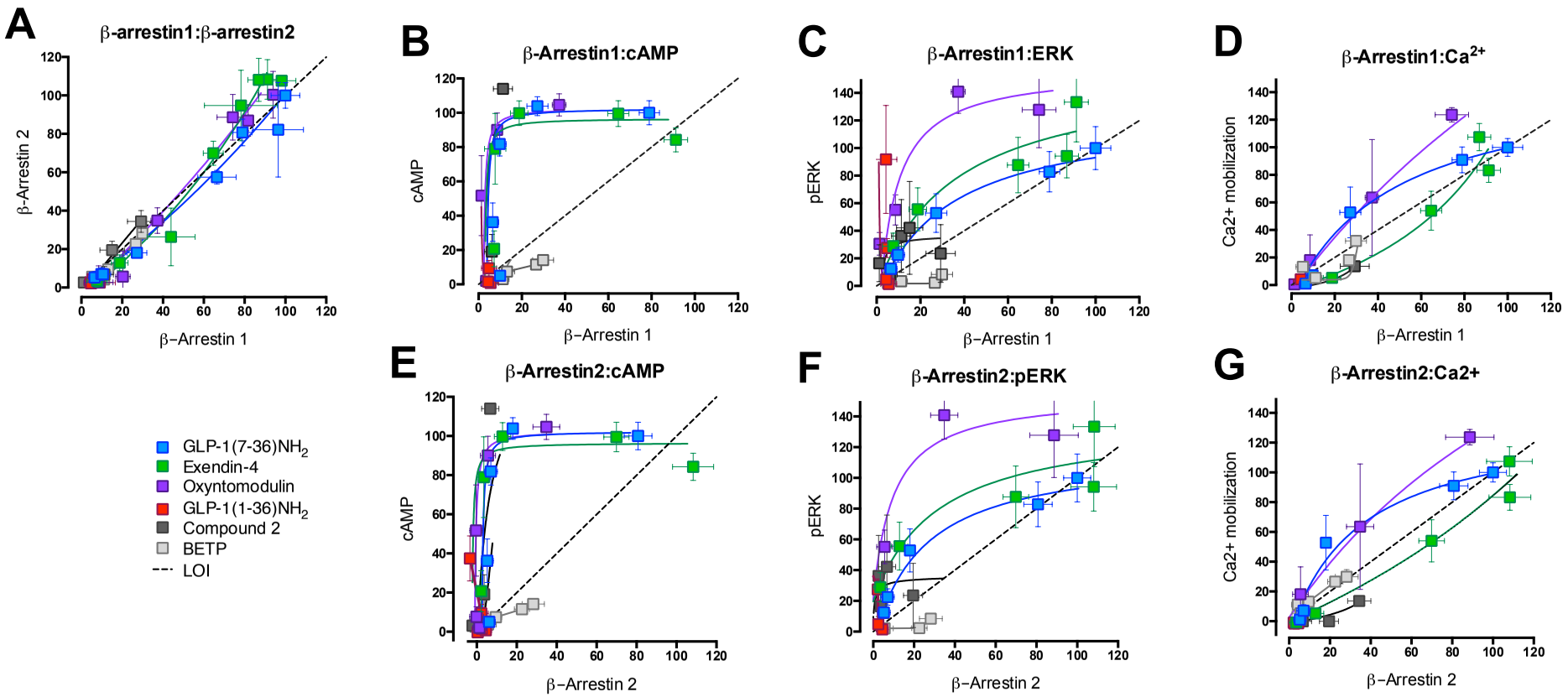

Figure 5.16. GLP-1R small molecules and peptides display stimulus bias relative to endogenous ligand GLP-1(7-36)NH_2.

Bias plots of β-Arrestin1 versus β-Arrestin2 (A), β-Arrestin1 versus cAMP (B), β-Arrestin1 versus pERK1/2 (C), β-Arrestin1 versus Ca^{2+} mobilization (D), β-Arrestin2 versus cAMP (E), β-Arrestin2 versus pERK1/2 (F) and β-Arrestin2 versus Ca^{2+} mobilization (G). Concentration response data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve. Equipotent concentrations of ligand and the curve derived from the analysis were then plotted against each other for each pathway combination. LOI = line of identity.

that describes equal bias for both pathways (Figure 5.16 A). With exception of BETP, all ligands are biased towards cAMP signalling over β-arrestin1 and β-arrestin2 recruitment (Figures 5.16 B and E). In addition, with the exception of BETP, all ligands are also biased towards pERK1/2 signalling over β-arrestin1 and β-arrestin2 recruitment (Figures. 5.16 C and F). However, relative to the primary endogenous agonist GLP-1(7-36)NH_2; GLP-1(1-36)NH_2, oxyntomodulin and exendin-4 more favorably couple the receptor towards the pERK1/2 signalling pathway in preference to β-arrestin1 and β-arrestin2 recruitment. In contrast, BETP is more biased towards β-arrestin1 and β-arrestin2. Interestingly, a distinct bias is observed between β-arrestin1 or -2 recruitment and Ca^{2+} mobilisation where oxyntomodulin, like GLP-1(7-36)NH_2, couples more favourably to Ca^{2+} mobilisation than β-arrestin2 recruitment, in this cell background. In contrast, exendin-4, Compound 2 and BETP more favourably couple to β-arrestin 1 and 2 recruitment (Figures. 5.15 D and G). BETP however lies on the line of identity (LOI) for Ca^{2+} mobilization:β-arrestin2, coupling equally well to Ca^{2+} mobilization and β-arrestin2, but compared to the reference ligand GLP-1(7-36)NH_2 it is biased towards β-arrestin2.

The relative bias between two signalling pathways can be quantified to allow statistical analysis of signal bias by applying equation 5 described in the materials and methods section Chapter 2. This can be applied to calculate a relative bias of individual ligands compared to a reference ligand (typically the primary endogenous ligand). As the analysis is compared to a reference ligand, the bias should apply independent of differential expression levels of signalling effectors in different cell backgrounds that may be assessed. In this study, the bias of each orthosteric peptide, Compound 2 and BETP has been compared to the reference ligand, in the case the GLP-1(7–36)NH_2 (Figure 5.17, Table 5.5). This revealed that GLP-1(1-36)NH_2 is significantly biased ($p < 0.05$) towards cAMP and pERK1/2 signalling pathways over β-arrestin1 and β-arrestin2 recruitment relative to GLP-1(7-36)NH_2 (Figures. 5.17, B, C, E and F; Table 5.5). In

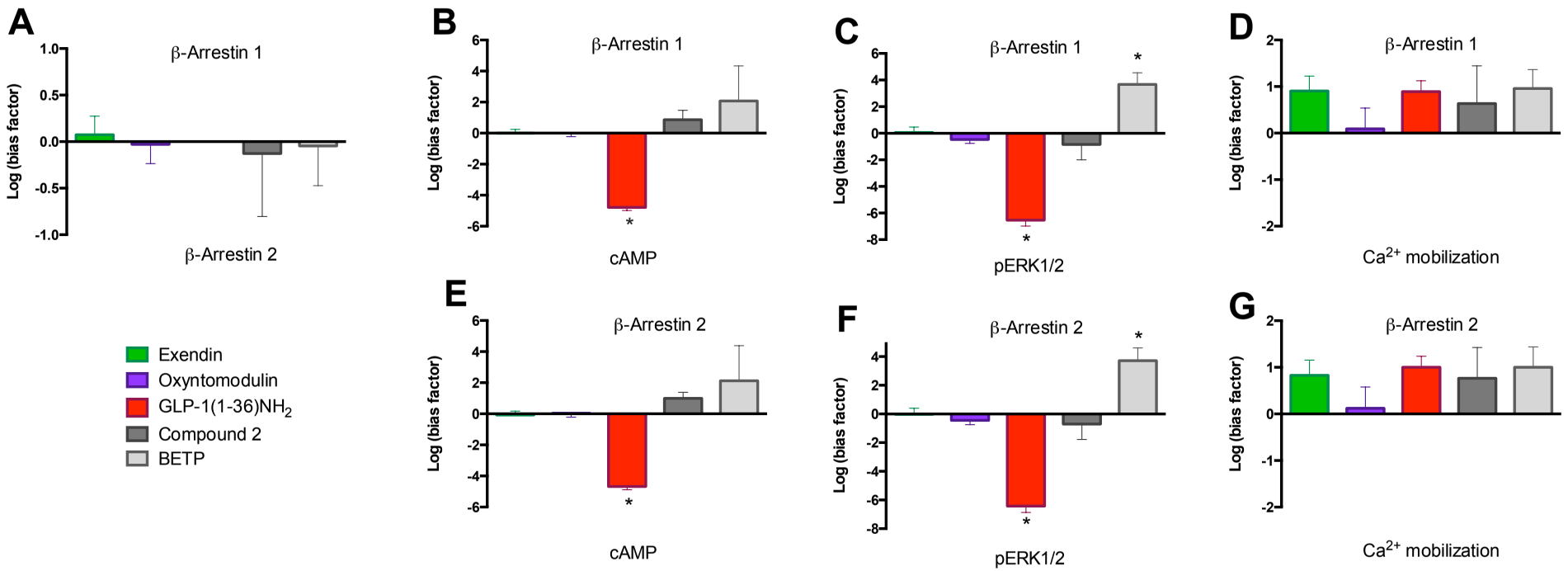

Figure 5.17. Biased signalling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.

Data were analysed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). β-Arrestin1 versus β-Arrestin2 (A), β-Arrestin1 versus cAMP (B), β-Arrestin1 versus pERK1/2 (C), β-Arrestin1 versus Ca^{2+} mobilization (D), β-Arrestin2 versus cAMP (E), β-Arrestin2 versus pERK1/2 (F) and β-Arrestin2 versus Ca^{2+} mobilization (G). Data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2. Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

Table 5.5. Biased Signaling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2

Data were analyzed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

Pathway: Pathway	GLP-1(7-36)NH_2	Exendin	Oxyntomodulin	GLP-1(1-36)NH_2	Compound 2	BETP
β-arrestin1: cAMP	0±0.19(1)	0.01±0.25(0.99)	0.00±0.23(1.01)	4.78±0.21(0.0)*		2.09±2.26(119.67)
β-arrestin1:pERK	0±0.31(1)	0.10±0.412(1.13)	0.473±0.29(0.34)	6.54±0.44(0.0)*	0.84±1.16(0.15)	3.68±0.87(4731.5)*
β-arrestin1: C^{a2+}	0±0.33(1)	0.91±0.32(8.02)	0.09±0.45(1.23)	0.89±0.23(7.78)	0.64±0.81(4.33)	0.96±0.41(9.06)
β-arrestin1:β-arrestin2	0±0.19(1)	0.074±0.20(1.19)	0.03±0.21(0.93)	0.11±0.13(0.78)	0.13±0.68(0.74)	0.05±0.43(0.90)
β-arrestin2: cAMP	0±0.20(1)	0.08±0.26(0.83)	0.033±0.24(1.08)	4.67±0.21(0.0)*	0.99±0.39(9.82)	2.12±2.26(133.06)
β-arrestin2: pERK	0±0.31(1)	0.02±0.42(0.95)	0.44±0.31(0.36)	6.43±0.45(0.0)*	0.71±1.06(0.20)	3.72±0.9(5260)*
β-arrestin2: C^{a2+}	0±0.33(1)	0.83±0.33(6.76)	0.12±0.46(1.32)	1.00±0.24(10.02)	0.76±0.66(5.81)	1.00±0.44(10.07)

addition, compared to GLP-1(7-36)NH_2, BETP significantly ($p < 0.05$) biases the receptor conformation towards β-arrestin1 and β-arrestin2 over pERK1/2 signalling (Figure 5.17; Table 5.5) Although all the ligands, with the exception of oxyntomodulin, bias the receptor conformation towards recruitment of β-arrestin1 and β-arrestin2 away from iCa^{2+} mobilization compared to GLP-1(7-36)NH_2, these values do not reach statistical significance.

Discussion

In this study we have characterised β-arrestin recruitment profiles induced by orthosteric peptides and small allosteric compounds that interact with the GLP-1R. In addition, we have assessed the ability of small molecules to modulate peptide-mediated β-arrestin recruitment, demonstrating that small ligands can differentially modulate the actions of larger peptide hormones. This study has identified further signalling bias induced by these compounds when compared with the endogenous peptide GLP-1(7-36)NH_2. As a result, we have expanded the signalling pathway repertoire covered in previous studies to include β-arrestin1 and β-arrestin2 recruitment to the GLP-1R. Understanding the signalling bias induced by distinct ligands, and their physiological effects downstream, allows us to begin to speculate on the importance of various pathways for targeting physiological functions, such as insulin secretion, appetite regulation and gastric emptying for therapeutic intervention for T2D.

Traditionally the role of β-arrestins in GPCR function is to act as terminators of G protein-mediated signalling, desensitizing the receptor signal and targeting receptors for internalisation. Although there is little literature on the role β-arrestins play in GLP-1R internalisation and desensitisation, there is evidence that upon activation by GLP-1(7-36)NH_2, GLP-1R internalisation can occur independently of β-arrestin recruitment and is not via a clathrin mediated mechanism (Al-Sabah et al. 2014). Instead, caveolin-1 regulates internalisation of the

GLP-1R when activated by GLP-1(7-36)NH_2 (Syme et al. 2006), these studies were performed in HEK-293 and insulinoma MIN6 cell lines.

In the past decade, there has been a plethora of information published showing that β-arrestins are not solely regulatory proteins that act to terminate GPCR signalling, but also mediate cellular signalling in their own right. To achieve this, they act as scaffolding proteins targeting multiple different proteins and bringing them together in complexes that mediate signalling events(Lefkowitz 2013b; Walther & Ferguson 2013; V. V. Gurevich et al. 2008; DeWire, Ahn, Lefkowitz & Shenoy 2007a). This makes these proteins attractive targets when developing new drugs for therapeutic intervention.

β-Arrestins present as a potential mediator of therapeutically important effects targeting symptoms associated with T2Ds, as they have been shown to act as scaffolding proteins that engender beneficial downstream outcomes including insulin release (Sonoda et al. 2008; Quoyer et al. 2010; M. Zhang et al. 2013a). β-Arrestin1 plays a role in GLP-1R-mediated pERK1/2 that enhances CREB activation and cAMP levels, subsequently contributing to insulin secretion (Quoyer et al. 2010; Sonoda et al. 2008). More recently, studies in β-arrestin2- deficient islets revealed that glucose-stimulated insulin secretion was decreased and the number of docked insulin filled granules was reduced compared to wild type islets, while insulin content and β-cell mass remained unchanged (M. Zhang et al. 2013a). Whether this is linked to GLP-1R or another mechanism is currently unknown.

Although current therapeutics for T2D are primarily directed towards increasing insulin secretion, not all engender other physiological effects that would be beneficial in long term treatment of T2D. This includes maintaining β-cell mass, by increasing β-cell proliferation and/or decreasing apoptosis. There is emerging evidence that targeting β-arrestin signalling downstream of the GLP-1R may provide some of these additional benefits, at least in the context

of rodent islets. In particular, β-arrestin1 signalling in β-cells targets Bad, MAPK, ERK, p90SK, all of which have been linked to anti-apoptotic effects (Quoyer et al. 2010). MAPK also plays an important role in controlling cell cycle, cell migration, cell proliferation and neogenesis(Roux & Blenis 2004). Furthermore, β-arrestin1-mediated activation of CREB has been linked to β-cell differentiation and proliferation (Quoyer et al. 2010; Sonoda et al. 2008). β-Arrestin1 is also able to form a β-arrestin-c-scr-GLP-1R complex that has been speculated to lead to β-cell proliferation (Talbot et al. 2012; Buteau et al. 2003). Biased GLP-1R ligands that target recruitment of β-arrestins are therefore likely to have downstream consequences such as activation of second messenger downstream signalling kinases. These could also mediate crosstalk, just as β-arrestin2 mediates crosstalk between β2AR and NF-κB signalling pathways that presents a novel mechanism for regulation of the immune system by the sympathetic nervous system (DeWire, Ahn, Lefkowitz & Shenoy 2007b; H. Gao et al. 2004).

As previously mentioned, there is now convincing evidence for the importance of β-arrestins in GLP-1R-mediated insulin secretion and β-cell survival. Studies from knock-out mice reveal the importance of β-arrestin1 in insulin secretion and studies in INS-1832 cells show that knockdown of β-arrestin1 results in decreased pERK1/2, CREB activation and reduced cAMP levels (Sonoda et al. 2008). In this study, we also investigated the ability of small non-peptidic compounds TT15, Boc5, and the 11-mer peptide agonist BMS21 to induce β-arrestin recruitment. Understanding the pharmacology of TT15 is of particular interest as a derivative of TT15 is currently in late phase 2 clinical trials and is therefore looking very promising as a potential therapeutic. Boc5, while not suitable for clinical development, is an intriguing tool to investigate GLP-1R-mediated biology due to its long plasma half life (>23.5 hrs.) (Ge et al. 2013), and its described effects in rodents, including induced durable restoration of glycemic control, weight loss, reduction in food intake and gastric emptying, increases in insulin secretion

and sensitivity (Su, He, Li, Liu, J. Wang, Y. Wang, W. Gao, Zhou, Liao, Young & M.-W. Wang 2008c). Although less is known about the physiological effects of BMS21, this compound has an enhanced pharmacokinetic profile compared to GLP-1, significantly reduces plasma glucose and is highly selective for the GLP-1R, albeit with an EC_{50} >10μM (Mapelli et al. 2009). Nonetheless, despite BMS21 having a much lower potency than GLP-1(7-36)NH_2, it has a higher efficacy for cAMP accumulation(Wootten et al. 2013). Interestingly, we were unable to detect any β-arrestin recruitment to the GLP-1R in response to any of these compounds. However, all of these compounds are capable of increasing cAMP accumulation and subsequent insulin secretion *in vivo* (D. Chen et al. 2007; Ge et al. 2013; Su, He, Li, Liu, J. Wang, Y. Wang, W. Gao, Zhou, Liao & Young 2008a; He et al. 2012; He et al. 2010; Q. Liu et al. 2012b; Gigoux & Fourmy 2013; Mapelli et al. 2009)(Rao, 2009)(Willard, Bueno, et al. 2012a). In addition, while not a drug-like compound, Boc5 also displays additional *in vivo* efficacies that would be promising for the treatment of T2D. For all these ligands, the concentrations assessed were high, however, all have low affinity for the receptor. It could therefore be argued that higher concentrations of these compounds may be required in our assays in order to be able to detect β-arrestin recruitment. Conversely, at the same concentrations of ligands used, we were able to detect responses to cAMP and less efficiently coupled pathways such as pERK1/2. Therefore, it is possible that recruitment of β-arrestins to the GLP-1R is not required for therapeutically relevant responses mediated by the GLP-1R. However, this does not necessarily mean that β-arrestin-mediated signalling is not involved and further studies are required to confirm this.

In this study, the peptide ligands GLP-1(7-36)NH_2 , exendin-4 and oxyntomodulin all recruited both β-arrestin 1 and β-arrestin 2 in a dose dependent manner in line with their affinities. The small molecules Compound 2 and BETP could also engender coupling of the receptor to both arrestins, however GLP-1(1-36)NH_2, Boc5, TT15 and BMS21 could not. This demonstrates

additional mechanistic distinction in the mode of action of the different classes of ligands that would be expected to lead to divergence in the pharmacological and indeed, clinical profile of the ligands.

Boc5 and TT15 modulate gastric emptying and food intake, in addition to promotion of insulin secretion (Su, He, Li, Liu, J. Wang, Y. Wang, W. Gao, Zhou, Liao & Young 2008a; D. Chen et al. 2007), suggesting that these effects are, at least in part, independent of arrestin dependent signalling. Nonetheless, the studies on arrestin recruitment to date have been restricted to CHO cells and the specific behaviour regulated by ligands is likely to be contextual on the levels of the proteins, inducing GRKs that have the potential to alter responses.

In our studies, we are measuring β-arrestin recruitment to the GLP-1R, but not directly β-arrestin activation. One specific study found an unanticipated mechanism for β-arrestin-mediated pERK1/2 responses following stimulation of the V2 vasopressin receptor (V2R)(Oligny-Longpré et al. 2012). For this receptor, β-arrestin2 is required for downstream pERK1/2 responses, and β-arrestin2 is strongly recruited to the V2R upon activation. However, the study revealed that it was not this pool of β-arrestin2 that was required for the pERK1/2. Activation of the V2R leads to stimulation of pERK1/2 through the metalloproteinase-mediated shedding of a factor activating the insulin-like growth factor receptor (IGFR). This possess is both Src- and β-arrestin2–dependent, where Src is activated downstream of the V2R, but upstream of metalloproteinase activation and is required for the release of the IGFR-activating factor. The involvement of β-arrestin2 was identified to occur downstream of IGFR transactivation, and it is therefore engagement of this pool of β-arrestin2 with the IGFR, but not that that interacts with the V2R that is required to promote the vasopressin-stimulated ERK1/2 activation (Oligny-Longpré et al. 2012). Therefore similar transactivation mechanisms may occur for other GPCRs and may explain some of the conflicting data around the importance of β-arrestin interactions

and β-arrestin-mediated signalling.

Although in our study we were unable to detect β-arrestin recruitment to the GLP-1R in response to TT15, Boc5 and BMS, this does not mean β-arrestin is not important in their ability to create physiological responses as it may function through other mechanisms that are independent of recruitment to the GLP-1R. One such mechanism is via a transactivation mechanism, like that described for the V2R. At this stage, more work is required to assess the mechanism via which β-arrestins are functioning to exert their downstream signalling consequences. It is also yet to be established for those ligands that can recruit β-arrestin to the GLP-1R, if this recruitment has any physiological relevance, or whether these ligands also promote β-arrestin-mediated physiological effects downstream of GLP-1R activation via a mechanism that is independent of β-arrestin recruitment to the GLP-1R. A recent study that may support this theory revealed that upon GLP-1(7-36)NH_2 activation, the GLP-1R could recruit β-arrestin2 to the receptor as measured using FRET. However, imaging using labeled β-arrestin2 and GLP-1R revealed that β-arrestin was recruited to the plasma membrane upon ligand activation, but while the receptor internalised, the β-arrestin remained at the surface. This may suggest that there is an additional pool of β-arrestin recruited to the cell surface that is not recruited to the GLP-1R, but to another transmembrane protein whose activity is dependent on GLP-1R activity (Noma et al. 2007; Oligny-Longpré et al. 2012; Buteau et al. 2003)

Alternatively, ligands that can recruit β-arrestins to the GLP-1R may implement differential mechanisms compared to ligands that cannot promote β-arrestin recruitment to the GLP-1R, to promote similar physiological effects. Studies on other family B GPCRs have identified different compounds that promote distinct signalling profiles to ultimately result in similar physiological outputs. One such example was at the PTH1R, whereby hPTH(1–34) activates solely G protein coupling and bPTH(7-34) antagonised receptor-G protein coupling but activated β-arrestin2

dependent signalling(Gesty-Palmer, Flannery, Yuan, Corsino, Spurney, Lefkowitz & Luttrell 2009b). However, *in vivo*, both ligands stimulated anabolic bone formation. The biased β-arrestin2 agonist primarily affected pathways that promoted expansion of the osteoblast pool, cell cycle regulation, cell survival and migration. In contrast, the endogenous agonist, PTH(1-34), primarily affected pathways classically associated with enhanced bone formation, including collagen synthesis and matrix mineralization that were less dependent on β-arrestin2 and more downstream of G protein activation. This study highlights how two PTH1R ligands with markedly different *in vitro* efficacy can elicit similar *in vivo* responses, and this concept may apply at the GLP-1R with different ligands having differential mechanisms (ie, arrestin-recruitment vs non-arrestin recruitment), leading to a similar physiological response(Gesty-Palmer, Flannery, Yuan, Corsino, Spurney, Lefkowitz & Luttrell 2009b).

In addition, this current study shows that allosteric modulation of these regulatory molecules is complex, with pathway-dependent modulation of receptor response that is determined by the combination of orthosteric ligand and allosteric ligand used. This emphasises the need for broad elucidation of mechanism of action and bias profiles when developing allosteric compounds.

Ligands that display signalling bias, whereby they preferentially activate some signalling pathways over others, have become a major focus in drug design, with ligand-mediated signal bias evident across a whole range of GPCRs. For example, for many years βAR antagonists (β-blockers) have provided many therapeutic benefits for patients with sustained acute myocardial infarction, and survival against heart failure. However more recently it has been found that the efficacy of different β-blockers varies widely. β-blockers have the ability to block G protein-mediated effects of excess catecholamine stimulation in the heart and other organs, thus becoming therapeutically relevant for a variety of cardiovascular conditions (Wisler et al. 2007).

Interestingly, carvedilol displays a unique profile with additional heart failure survival advantages compared to other β-blockers. Carvediol acts to antagonize G-protein-mediated signalling while simultaneously stimulating β-arrestin–mediated signalling (Wisler et al. 2007). This biased activity towards the β-arrestin pathway at the β1-AR with no G-protein signalling may have greater value for cardioprotection compared to propranolol, which only blocks G protein-mediated signalling, but is not an agonist for β-arrestin (Correll & McKittrick 2014; Wisler et al. 2007). In addition, selectively engaging β-arrestins at the AT1R may provide a therapeutic advantage for hypertension as this mechanism reduces blood pressure and increases cardiac performance (Violin et al. 2010). It has also been speculated that D2R β-arrestin2 biased ligands may have some potential for neurological diseases as they provide beneficial antipsychotic activity while simultaneously providing protection against motoric side effects (Allen et al. 2011).

Due to the emerging roles of GLP-1R mediated β-arrestin signalling in insulin secretion, β-cell proliferation, differentiation and apoptosis, GLP-1R ligands that bias response towards β-arrestin signalling could potentially be of therapeutic relevance as they may lead to an increased β-cell mass due to enhancing β-cell proliferation, differentiation and the antiapoptotic effects that occur downstream of β-arrestin1-mediated MAPK activation. They may also increase in insulin secretion as a result of β-arrestin1 or β-arrestin2-mediated increases in insulin-docked granules.

In the current study, assessment of signalling across five pathways (cAMP, pERK1/2, iCa^{2+} mobilization, β-arrestin1 and β-arrestin2 recruitment) with multiple ligands demonstrates that all ligands are biased in their signalling profile compared to GLP-1(7-36)NH_2. The GLP-1R when activated by GLP-1(7-36)NH_2 most strongly couples to cAMP, with lower efficacy in pERK1/2, iCa^{2+} mobilisation and β-arrestin recruitment. While this is also true for the other ligands (with

the exception of BETP), there is relative bias between cAMP and these other pathways across the different ligands (Figure 5.15). In addition, there is also bias between each of the other four pathways assessed (Figure 5.16). In combination with previous work, we revealed that GLP-1(1-36)NH_2 was significantly more bias towards cAMP and pERK1/2 signalling over β-arrestin1 and β-arrestin2 relative to GLP-1(7-36)NH_2, whereas BETP displayed significant bias towards both β-arrestin1 and β-arrestin2 over pERK1/2 signalling relative to GLP-1(7-36)NH_2 (Figure 5.17, Table 5.5). Integrating the current study with previous work, Compound 2 displays significant bias with less preference for cAMP signalling relative to iCa^{2+} mobilization, β-arrestin1 and β-arrestin2. In contrast, BETP displayed a very different profile to GLP-1(7-36)NH_2; not only does BETP display bias towards iCa^{2+} mobilization as previously reported, but also displays strong biased towards β-arrestin recruitment, relative to cAMP and pERK1/2 signalling. Furthermore, this response is biased towards β-arrestin1 recruitment and iCa^{2+} mobilization over β-arrestin2 (Figure 5.17; Table 5.5). However one important finding highlights the tendency of most if not all ligands to stimulate cAMP production with much higher potencies than other signaling pathways. Thus, it seems that the receptor itself is more efficiently coupled to Gs than to other pathways, and that for most ligands, AC activation will be the predominant biochemical outcome. Thus, it is not surprising that ligands that produce differentially biased signaling do not always produce distinct outcomes *in vivo*.

This ligand-mediated biased signalling concept also extends to allosteric modulation of orthosteric ligand responses. As previously highlighted, small molecules can display differential intrinsic efficacy profiles, if they bind allosterically, they can also differentially modulate peptide (both endogenous and exogenous) responses in a pathway specific manner. Thus, identifying the modulatory profile of small molecules Compound 2 and BETP using multiple functional outputs in conjunction with different GLP-1R orthosteric ligands is important, especially when this

endogenous system involves the interplay of many natural ligands and several signalling pathways to elicit physiological consequences.

The small allosteric molecule, Compound 2 has been previously characterized in cAMP accumulation, pERK1/2, and iCa^{2+} mobilisation assays (Table 5.5) (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010b; Coopman et al. 2010). This previously published work demonstrates that Compound 2 imparts significant bias towards cAMP accumulation mediated by oxyntomodulin, however there was no bias towards pERK1/2 signalling or Ca^{2+} mobilization. For the other peptides neutral cooperativity was observed for exendin-4, GLP-1(7-36)NH_2 and GLP-1(1-36)NH_2 in the aforementioned pathways revealing probe dependence in this effect. Furthermore, significant negative cooperativity between Compound 2 and exendin-4-mediated pERK1/2 responses was observed with a similar trend for both GLP-1(7-36)NH_2, GLP-1(1-36)NH_2 and oxyntomodulin. This probe-dependent interaction between Compound 2 and peptide agonists extended into the current study, where Compound 2 displayed positive cooperativity with exendin-4, GLP-1(7-36)NH_2 and oxyntomodulin for recruitment of both β-arrestin1 and β-arrestin2, but the observed cooperativity was significantly ($p < 0.05$) greater for GLP-1(7-36)NH_2 and oxyntomodulin, than for that of exendin-4 (Figure 5.10 A-C, 5.11 A-C; Table 5.5).

GLP-1(1-36)NH_2 did not display any agonism on its own in the recruitment of β-arrestins, and neither BETP or Compound 2 were able to induce a response at the concentrations tested (Figure 5.9 D, 5.10 D; Table 5.4).

Assessment of BETP interactions with peptide ligands revealed neutral cooperativity between BETP and exendin-4 for recruitment of both β-arrestin1 and β-arrestin2 (Figure 5.12 B, 5.13 B; Table 5.4). In contrast, while neutral cooperativity was observed between BETP and GLP-1(7-36)NH_2 for β-Arrestin1 recruitment (Figure 5.12 A Table 5.4), BETP weakly potentiated GLP-

1(7-36)NH2-mediated β-Arrestin2 recruitment (Figure 5.13 A; Table 5.4). There was also weak potentiation of oxyntomodulin-induced recruitment of both β-arrestin1 & β-arrestin2 (Figure 5.12 C, 13 C; Table 5.4) (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Wootten et al. 2013).

Another behaviour explored in the current work was the ability of the allosteric ligands to cause marked potentiation of previously "inert" metabolic products in a pathway specific manner. In chapter 4, we investigated the potential to allosterically modulate the activity of the "inactive" metabolite GLP-1(9-36)NH_2 at the GLP-1R. Briefly, these findings showed that BETP and Compound 2 were able to strongly potentiate cAMP signalling in heterologous cell systems. Furthermore this study demonstrated that 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. In the current chapter the potential of both small allosteric compounds to modulate GLP-1(9-36)NH_2-mediated recruitment of β-arrestin1 was investigated, as β-arrestin1 has been reported to be essential for activation of signalling pathways leading to insulin secretion downstream of GLP-1R activation (Sonoda et al. 2008; Quoyer et al. 2010). Similar to observations in cAMP and insulin secretion studies, increasing concentrations of either Compound 2 or BETP potentiated GLP-1(9-36)NH_2-mediated β-arrestin1 recruitment, however the effect was modest compared to the effect seen for cAMP production, (chapter 4) (Figure 5.15 A-B).

Collectively, the work has identified a large array of different signalling profiles for ligands that show bias towards particular signalling pathway on their own and a different profile when co-bound with an allosteric modulator. In order to draw conclusions and interpret this work it is necessary to not only detect levels of β-arrestin recruitment, cAMP accumulation, pERK1/2 and iCa^{2+} mobilization but to look at different functional outputs. It is well know that cAMP

accumulation is closely linked to insulin secretion, however little is known about the source of cAMP and whether it is compartmentalized and derived from multiple signalling pathways. In addition, β-arrestins are scaffolding proteins and are not limited to receptor regulation. Although studies have highlighted the importance of β-arrestins in β-cell function, modulation of these proteins by small allosteric compounds needs further investigation in terms of the β-cell physiological outputs such as; β-cell proliferation, differentiation, apoptosis and insulin secretion. It is difficult to speculate on the ideal combination of ligands and which pathways are more beneficial to modulate over others. These studies have identified many different signalling profiles however, in order to fully understand and tailor the most effective treatment for T2Ds, these endpoints need to be further investigated in a more physiological setting.

The aforementioned studies reveal that GLP-1R conformations induced by co-binding of an allosteric modulator and orthosteric ligand can vastly alter the combined signalling profile of the receptor such that no two combinations of allosteric-orthosteric ligand pair were able to produce the same profile of behavior. A recent study revealed that both Compound 2 and BETP share a common binding pocket, both covalently modifying a cysteine residue (Cys^{347}) in ICL3 of the GLP-1R (Nolte et al. 2014). However, the specific interaction formed between these compounds and the receptor are clearly different as they induce very distinct bias in their efficacy and modulatory properties.

The ability of individual ligands to differentially activate the GLP-1R to produce distinct functional profiles by altering one pathway while simultaneously stimulating different effects on another pathway, as well as differential probe-dependent effects may provide a unique opportunity in drug development. This offers the potential to sculpt receptor signalling to target physiologically important responses and exclude those that do not provide beneficial outputs. However, despite this therapeutic potential, the complexity of intracellular signalling presents

challenges as it is currently unclear what the ultimate pathway or combination of pathways that need to be manipulated in order to drive the ideal therapeutic responses. This chapter investigates compounds that display differential efficacy and modulatory profiles, thus providing us with tools that could further develop our understanding regarding the physiological consequences of biased signalling *in vivo/ex* vivo settings. Further work is necessary to understand these concepts and delineate the ideal signalling profile for future therapeutics.

In conclusion, we have demonstrated that, together with previously published work, these small molecule ligands induce biased signalling across multiple pathways at the GLP-1R and also provide additional evidence of the probe-dependent nature of small allosterically acting molecules. Further work is required to delineate the extent to which such bias exists in a native cellular environment and the *in vivo* consequences. This work highlights the importance of understanding the full signalling repertoire of small molecule GLP-1R ligands as they may have the potential to sculpt signalling for greater selectivity and improved therapeutic outcomes. In the context of pleiotropically coupled receptors and the interplay of multiple pathways leading to physiologic responses, profiling of small molecules in this manner may lead to a better understanding of the physiologic consequences of biased signalling at this receptor. This could enable the design and development of improved therapeutics that have the ability to fine-tune receptor signalling, leading to beneficial therapeutic outcomes while reducing side effects profiles.

CHAPTER 6:

Ligand mediated G protein-receptor kinase interactions, allostery and biased signalling at the GLP-1R

Introduction

GPCRs are involved in the regulation of a wide variety of important physiological processes. The classic paradigm of GPCR signal transduction processes involves ligand-induced conformational changes in the receptor that results in the generation of intracellular signals and also initiates receptor desensitisation and internalisation (E. V. Gurevich et al. 2012; Mushegian et al. 2012). Just as G proteins recognize activated receptors, G protein-coupled receptor kinases (GRKs) also recognize activated GPCRs, which leads to catalytic activation of these protein kinases. Following ligand binding to the GPCR, GRKs are recruited to the cell membrane where they phosphorylate specific residues located in the intracellular loops and carboxyl terminus of the activated receptor. Receptor phosphorylation subsequently promotes high affinity binding of β-arrestins to the receptor, hindering further coupling of G proteins and significantly diminishing receptor signalling(Lohse & Hoffmann 2014; Goodman et al. 1996). Following this, the classical paradigm of β-arrestin interaction results in receptor desensitisation /internalisation. Recent studies have also revealed a role for GRKs in β-arrestin-independent signalling events that require initial GRK-catalyzed phosphorylation of receptors (Kohout & Lefkowitz 2003; Penela et al. 2006).

Many GPCRs undergo desensitisation that requires precise coordinated actions of GRKs and arrestins, thus preventing the potentially harmful effects to the cell that can result from persistent receptor stimulation(Magalhaes et al. 2012). There are seven GRKs, four of which are ubiquitously expressed throughout the body. GRK2 and related GRK3 share a carboxyl-terminal pleckstrin homology domain and are recruited to the plasma membrane via G protein βγ-subunit-mediated translocation. The recruitment of the βγ-subunit is absent for GRK5 and GRK6 and instead they use direct PIP_2 binding and/or covalent lipid modification with palmitate to reside primarily at the plasma membrane (Pitcher et al. 1996; Pitcher et al. 1995).

Until recently there was little understanding of the diverse involvement of GRKs in GPCR signalling. In particular, different tissues and cells can exhibit differential GPCR phosphorylation patterns, an example of which is evident for the M3 mAChR (Butcher et al. 2011). In addition, GRK2 and GRK6 phosphorylate different sites on the β2-AR, which determines the downstream signalling consequences following β-arrestin recruitment to the receptor, this may result in β-arrestin-mediated desensitization of receptor signalling or G protein independent signalling respectively (Nobles et al. 2011). Furthermore, GRK2 and GRK3 promote V_2R desensitization, while GRK5 and GRK6 are responsible for ERK1/2 activation via this receptor (Ren et al. 2005). Similarly phosphorylation of the $AT_{1A}R$ by GRK2 and GRK3 induces desensitization and internalization, whereas phosphorylation by GRK5 leads to β-arrestin-dependent ERK1/2 activation (J. Kim et al. 2005; Watari et al. 2014). These examples demonstrate how different GRKs can promote a diverse range of functions following GPCR activation.

Intracellular signalling mediated by multiple ligands (both orthosteric and allosteric) of the GLP-1R have been characterised for cAMP accumulation, ERK1/2 phosphorylation, iCa^{2+} mobilization, β-arrestin1 and β-arrestin2 recruitment as discussed in chapters 1 and 4 (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Wootten et al. 2013; Willard, Wootten, et al. 2012b; Coopman et al. 2010; Selway et al. 2012). This study builds on the already existing data exploring signal bias at the GLP-1R, to incorporate recruitment of GRK2, -3, -5 and -6, and to explore their roles in β-arrestin recruitment to the GLP-1R.

Studies on the GLP-1R have used fusion proteins to show involvement of GRK5 in β-arrestin2 recruitment in response to GLP-1(7-36)NH_2 and glucagon (Jorgensen et al. 2005). Additionally,

there is some indication that oxyntomodulin stimulation mediates recruitment of GRK2 to the GLP-1R(Jorgensen et al. 2007). However, to date, there is no comprehensive analysis of ligand-mediated recruitment of GRKs to the GLP-1R. Furthermore, there is little understanding of the importance of distinct GRKs in GLP-1R signalling or regulation. Given the emerging roles of GRKs in multiple signalling mediated events, in addition to receptor regulation, it is important to understand the signalling profile of different ligands that act at the same GPCR, as ligand-mediated recruitment/activation of certain GRKs may determine either a positive or negative physiological outcome. This also applies when there is more than one ligand occupying the receptor at one given time, specifically, the presence of an allosteric ligand that has the potential to modulate specific pathways at the expense of others. The previous chapter revealed that two allosteric modulators of the GLP-1R, Compound 2 and BETP, could mediate biased recruitment of arrestins, compared to peptide ligands. In addition, these ligands could also induce differential bias into the system with respect to β-arrestins when co-bound to the receptor with different orthosteric ligands.

It is important to elucidate how GRKs interact with the GLP-1R, whether they promote GPCR desensitization or G protein-independent signalling and the mechanism by which they do this. In order to begin to answer these questions, it is important to profile different ligands and their ability to recruit specific GRKs. In addition, it is important to establish how allosteric ligands modulate recruitment of GRKs, both alone and in conjunction with different peptide ligands.

In this chapter, the same BRET system employed in the previous chapter was used to investigate GRK 2,3,5 and 6 recruitment to the GLP-1R in a CHOFlpIn cell line. As highlighted in the previous chapter, probe dependence and signalling bias are two phenomena that are attracting increased attention in the study of receptor structure/function studies. This work further builds on

previous work from Chapter 5, investing the recruitment profile of different GRKs by distinct orthosteric ligands in the presence and absence of small allosteric molecules Compound 2 and BETP at the GLP-1R.

Results

GRKs are recruited to the GLP-1R when activated by different classes of ligand

We used the BRET method described in chapter 4 to assess GRK recruitment to the GLP-1R. To determine this, the β-arrestin1-Venus in the dual expression vector was replaced with GRK2-venus, GRK3-venus, GRK5-venus or GRK6-venus. GRK1, GRK4 and GRK7 were excluded from this study, as GRK1 and GRK7 are largely limited to vertebrate rod and cone photoreceptors, whereas GRK4 is only highly expressed in the testis (Reiter & Lefkowitz 2006b; Willets, Challiss & Nahorski 2003b; Sallese et al. 1997; Erdtmann-Vourliotis et al. 2001).

As with previous studies, the following ligands were assessed for their ability to recruit each GRK to the GLP-1R; GLP-1(7-36)NH_2, exendin-4, oxyntomodulin, GLP-1(1-36)NH_2, Compound 2, BETP, Boc5, BMS21 and TT15. To determine if the ligands recruited the various GRKs, kinetic experiments were performed whereby the BRET profile was assessed for 15 min following ligand addition.

GRK2 is weakly recruited to the GLP-1R following ligand stimulation and Compound 2, but not BETP can enhance peptide-mediated recruitment.

Following addition, GLP-1(7-36)NH_2 (100 nM), exendin-4 (100 nM) and oxyntomodulin (1 μM), were able to induce very weak recruitment of GRK2 (Figure 6.1 A-C). 1 μM GLP-1(1-36)NH_2 did not recruit GRK2 to the GLP-1R (Figure 6.1 D). Compound 2 (1 μM) also displayed very weak recruitment GRK2 when added alone, and when co-added with peptides enhanced GRK2 recruitment mediated by GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin. GLP-1(1-36)NH_2 was unable to recruit detectable levels of GRK even in the presence of Compound 2.

BETP (1 μM) induced a larger BRET signal for GRK2 recruitment than any of the peptide ligands assessed or Compound 2. However, when 1 μM BETP was co added with each of the

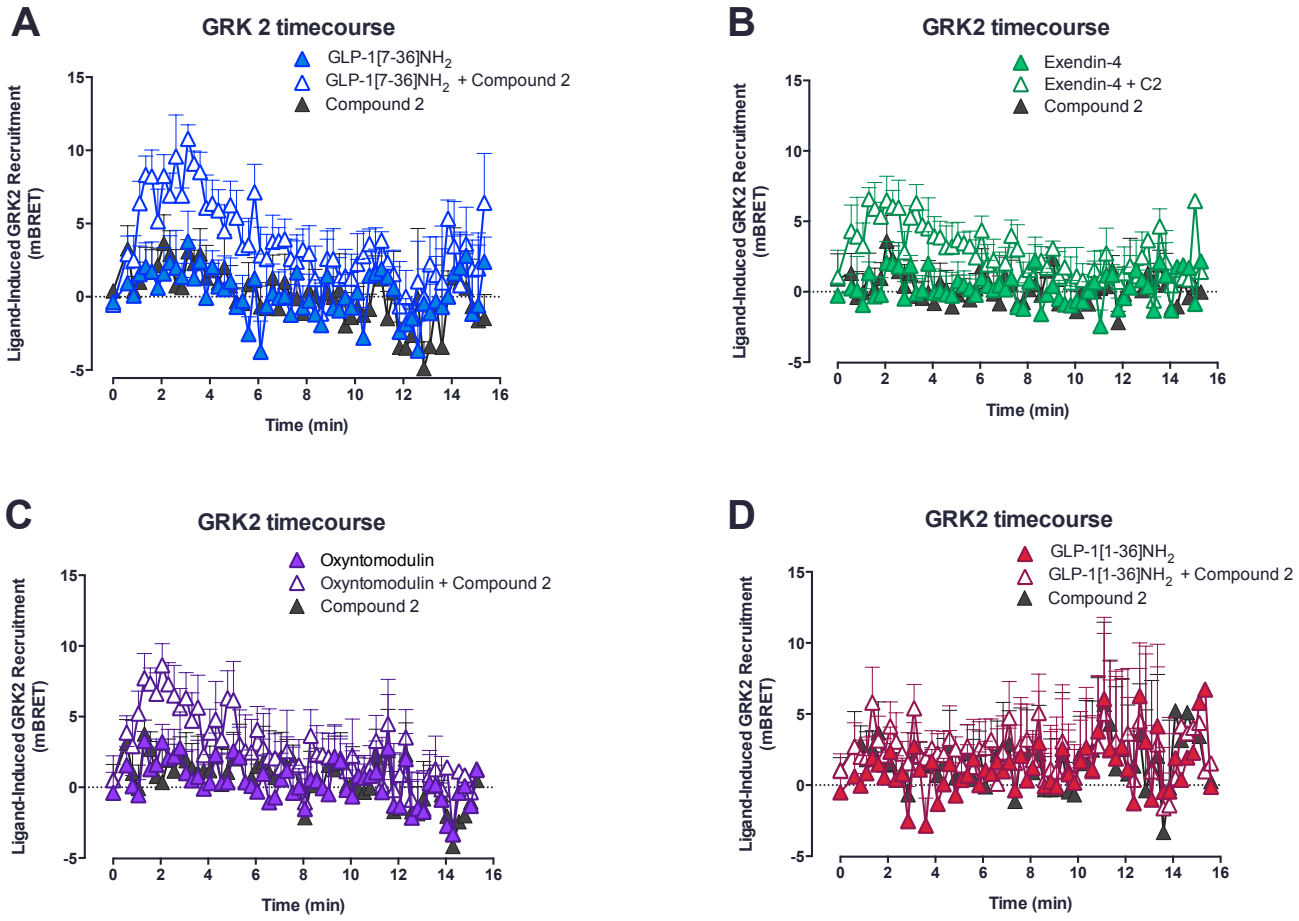

Figure 6.1 GRK2 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

peptide ligands, there was no significant increase in GRK2 recruitment as these traces matched that of BETP alone (Figure 6.2 D). The other synthetic ligands; Boc5 (10 μM), TT15 (10 μM) and BMS21 (1 μM) were unable to recruit GRK2 to the GLP-1R over the timecourse and concentration tested (Table 6.2).

Overall, the kinetic profile of ligand-mediated GRK2 recruitment was consistent across ligands with the response peaking at approximately 2.5 - 3 min and remaining sustained (but with a gradual drop over time) throughout the 15 min timecourse. Interestingly, the co-addition of 1 μM Compound 2 increased the transient recruitment of GRK2 that was mediated by peptide alone with an increased response at 2.5-3 min that rapidly dropped back to peptide or Compound 2 alone by 5-6 min. This peak response was enhanced for GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin by 55%, 66% and 71% above the peptide response alone, respectively (Figure 6.1 A-C).

GRK3 is selectively recruited to the GLP-1R by distinct ligands and recruitment by peptides is differentially modulated by the co-addition of allosteric ligands. Investigation of the recruitment of GRK3 to the GLP-1R revealed that following addition of either GLP-1(7-36)NH_2 (100 nM) or oxyntomodulin (1 μM), there was transient recruitment of GRK3 that peaked 2 minutes post addition (Figure 6.3 A, C). This transient peak dropped to a sustained recruitment that was observed above the vehicle control. Similarly, exendin-mediated recruitment also peaked at 2.5 min, however this response was much weaker than that observed with GLP-1(7-36)NH_2 and oxyntomodulin (40% of the GLP-1(7-36)NH_2 response) (Figure 6.3 B). In contrast to GRK2, GLP-1(1-36)NH_2 was able recruit GRK3, however the magnitude of effect was very weak compared to the response observed for the other peptides, and this response was sustained after peaking at 2.5 min. (Figure 6.3 D). The response in mBRET units was greater for all

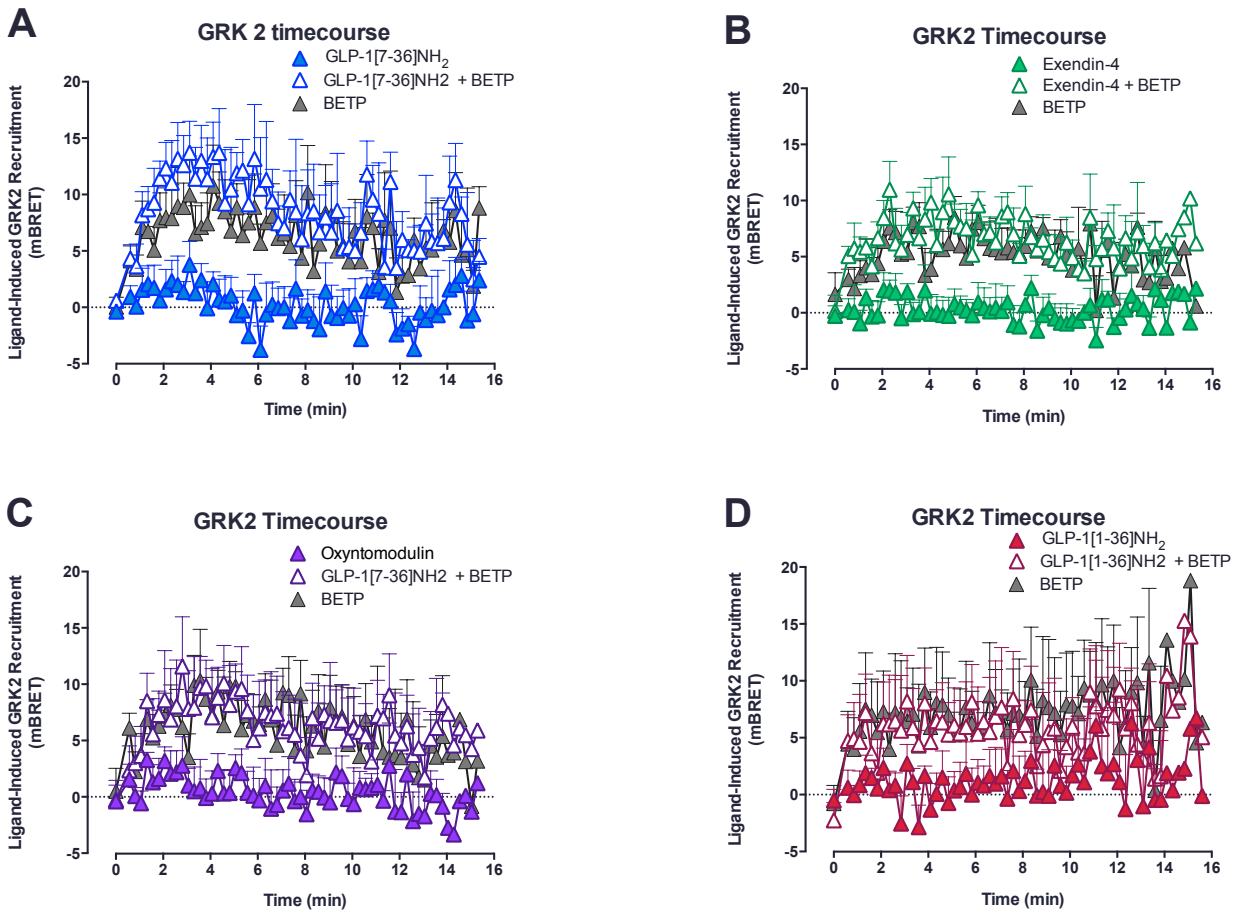

Figure 6.2. GRK2 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

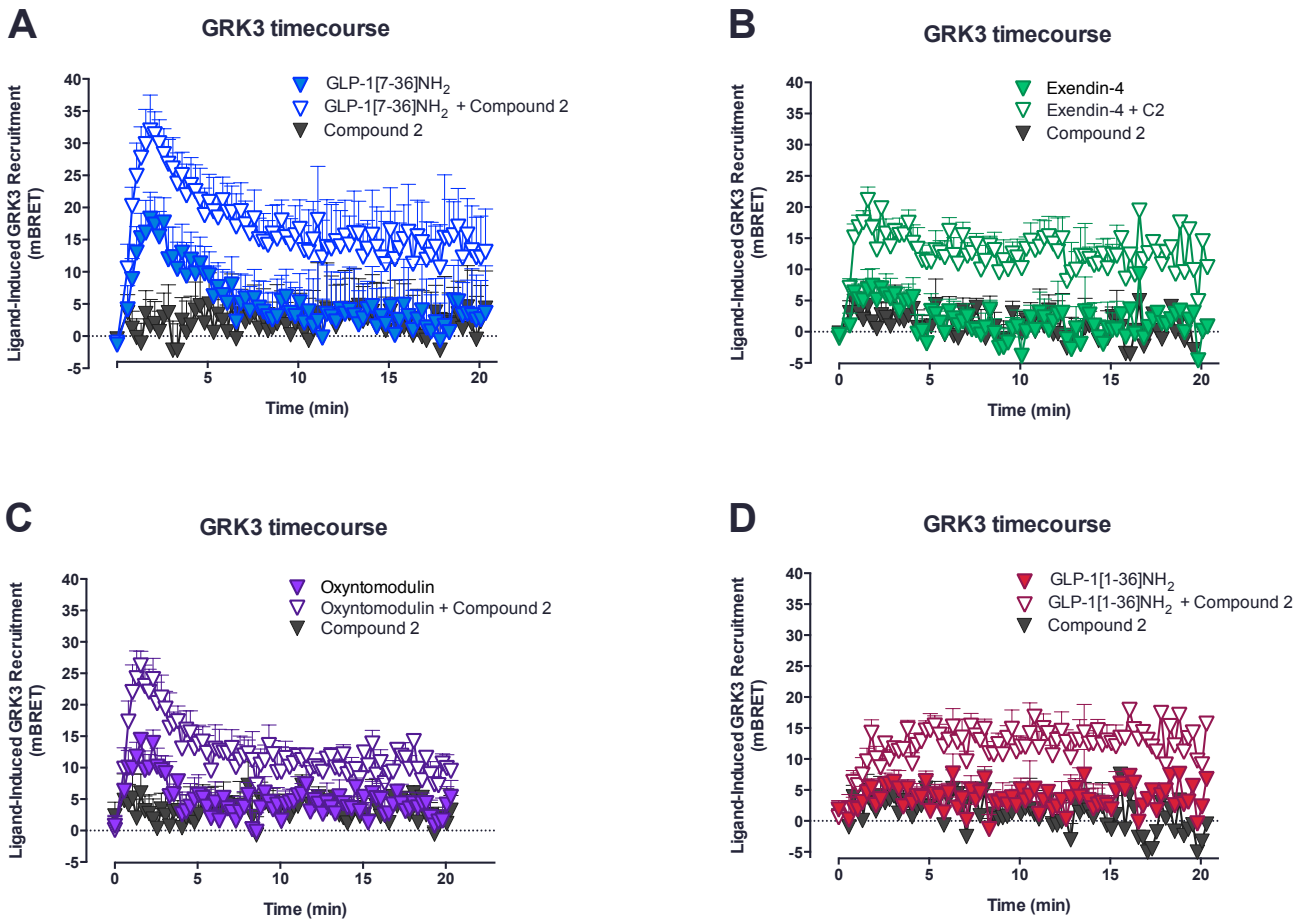

Figure 6.3. GRK3 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator Compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

peptides for GRK3 than that observed for GRK2, as GRK2 responses in the absence of coaddition of allosteric ligands were only weakly detectable above vehicle alone.

Compound 2 was only able to promote weak recruitment of GRK3 to the GLP-1R when added alone, however, this compound enhanced GLP-1(7-36)NH_2-mediated recruitment of GRK3 by 43% above peptide response and this response remained elevated throughout the entire timecourse (Figure 6.3 A). This response showed the same profile as peptide alone with a transient peak that dropped to a lower sustained response. Furthermore, Compound 2 also enhanced recruitment of GRK3 when co-added with oxyntomodulin with a 46% increase in response above the peptide alone in the transient peak and an elevated sustained response (Figure 6.3C). Co-addition of Compound 2 also increased exendin-4-mediated recruitment of GRK3, however, the kinetics of this were distinct from GLP-1(7-36)NH_2 and oxyntomodulin (Figure 6.3B). The recruitment was enhanced by 66% above peptide response alone, and was elevated throughout the timecourse of the experiment, with no response decay observed (Figure 6.3 B). Similarly, co-addition of Compound 2 and GLP-1(1-36)NH_2 displayed recruitment of GRK3 of 15 mBRET above the basal response with a kinetic profile similar to exendin-4 (sustained recruitment over the time-course).

In comparison to Compound 2-mediated recruitment of GRK3, BETP displayed rapid recruitment of GRK3 to the GLP-1R, with a response that peaked at 3 minutes post ligand addition and was sustained for most of the timecourse (Figure 6.4). This response was weaker than that observed for GLP-1(7-36)NH_2 and oxyntomodulin, but greater than exendin-4 and GLP-1(1-36)NH_2 (Figure 6.4). Peptide mediated GRK3 recruitment was also investigated in the presence of BETP. BETP only enhanced GLP-1(7-36)NH_2 and oxyntomodulin-mediated recruitment of GRK3 by 25 % and 32 % above peptide respectively (Figure 6.4 A). Interestingly,

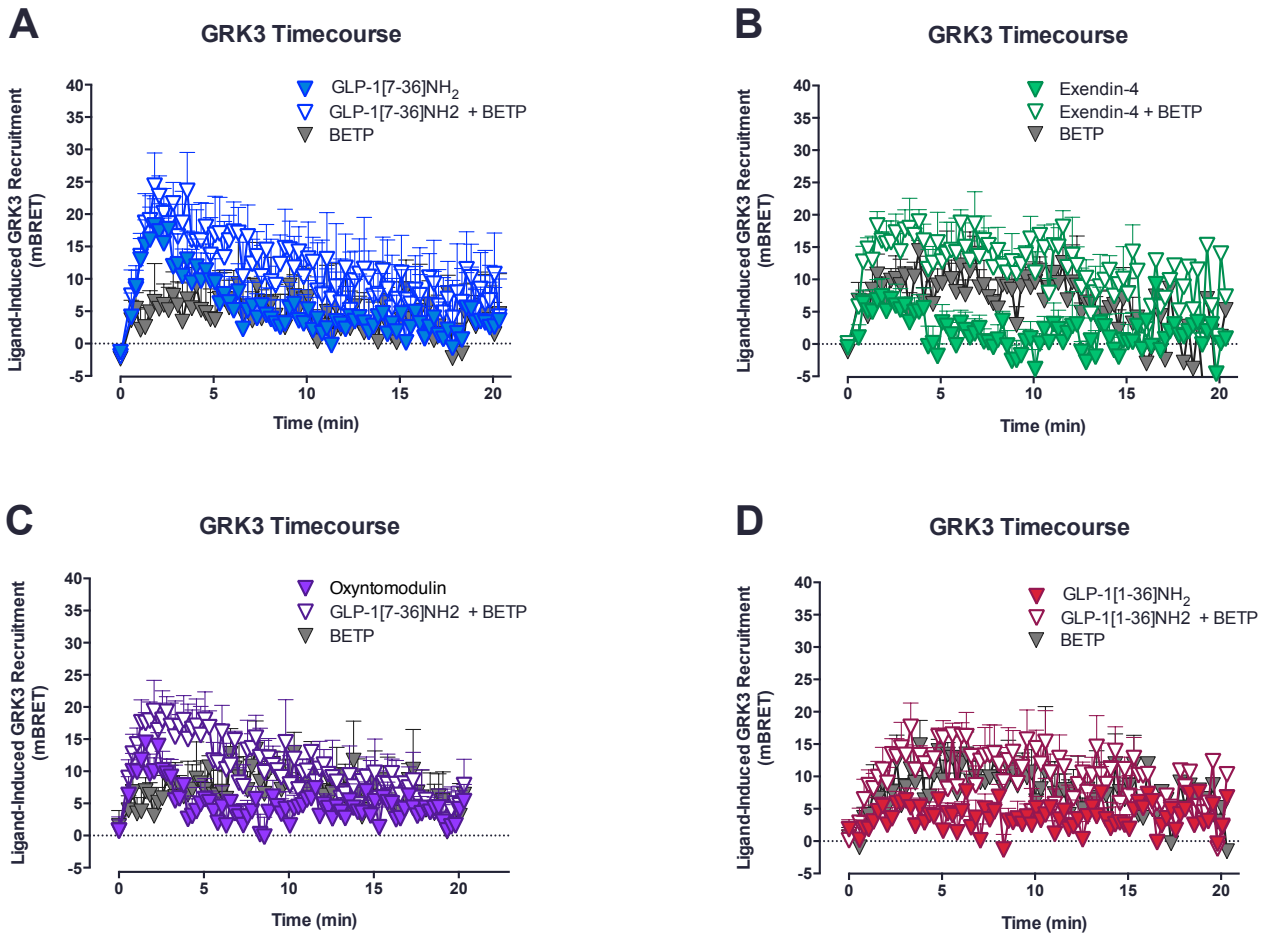

Figure 6.4 GRK3 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

this coaddition resulted in a longer sustained response, compared to peptide alone. Co-addition of BETP and exendin-4 did not significantly enhance GRK3 recruitment observed by BETP alone (Figure 6.4 B). In addition, there was no increase in GRK3 recruitment in the presence of GLP-1(1-36)NH2 (Figure 6.4 D). Similar to that observed with GRK2, the synthetic ligands Boc5 (10 μM), TT15 (10 μM) and BMS21 (1 μM) were unable to recruit GRK3 to the GLP-1R at the concentration of ligands tested (Table 6.2).

GRK5 displayed a unique peptide-mediated recruitment profile to the GLP-1R in comparison to GRKs 2 and 3, which was altered by coaddition of BETP but not Compound 2.

GRK5 displayed a very different recruitment profile in response to each GLP-1R peptide ligand in comparison to GRK2 and GRK3. Similar to GRKs 2 and 3, each peptide initially displayed a very rapid (albeit weak) recruitment of GRK5 that peaked at 40-45 seconds. However, in contrast, following this transient peak, all responses dropped below recorded basal levels where they plateaued around 7-13 mBRET units below baseline depending on the peptide (Figure 6.5-6.6).

Interestingly, GLP-1R stimulation by Compound 2 displayed weak recruitment at 2.5 min that dropped back to baseline but did not fall significantly below this over the timecourse of the experiment. Co-addition of Compound 2 with peptide had little effect on the recruitment profile induced by any of the peptides (Figure 6.5).

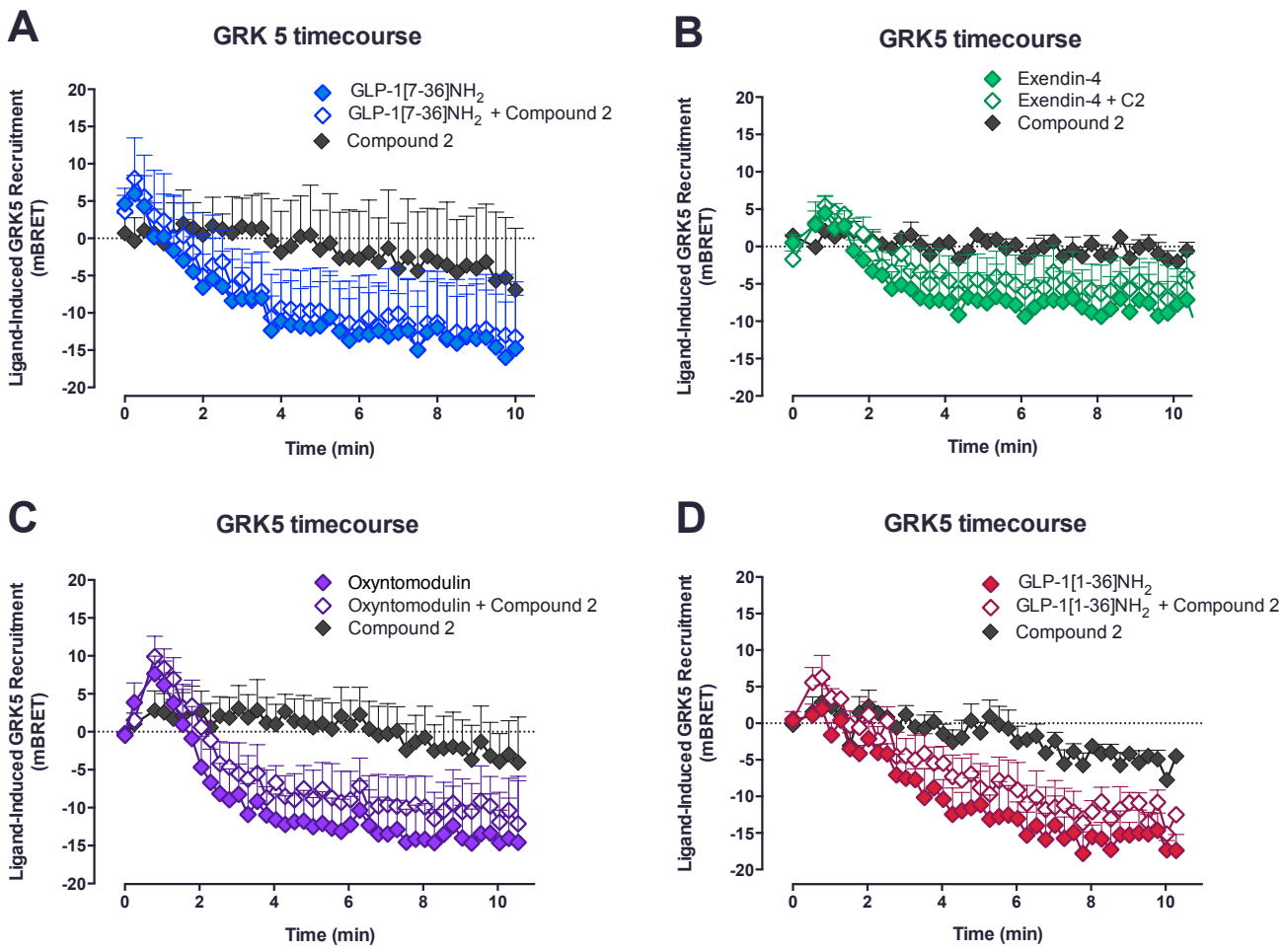

Figure 6.5 GRK5 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), mediated recruitment of GRK5 to the GLP-1R in the presence and absence of GLP-1R small molecule allosteric modulator Compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

BETP did not show any significant change in the recruitment profile for GRK5 to the GLP-1R compared to vehicle alone. However, unlike Compound 2, 1 μM BETP altered the kinetic profile of all peptide responses. Although the peak response was unaltered, the drop below baseline, was almost entirely lost for exendin-4, oxyntomodulin and GLP-1(1-36)NH_2, with the peak response returning to within basal levels of BRET response (Figure 6.6 B-D). GLP-1(7-36)NH_2 displayed a similar trend, although was not as pronounced (Figure 6.6 A).

The synthetic ligands Boc5 (10 μM), TT15 (10 μM) and BMS21 (1 μM) did not recruit detectable levels of GRK5 to the GLP-1R at the concentration of ligands tested (Table. 6.2).

Selective peptide-mediated GRK6 recruitment profiles were similar to GRK5 but displayed differential patterns by coaddition of BETP compared to Compound 2.

Ligand-mediated GRK6 recruitment to the GLP-1R was very poor, with no detectable peak for recruitment of GRK6 in response to GLP-1(7-36)NH_2, exendin-4, GLP-1(1-36)NH_2, oxyntomodulin or Compound 2 (Figure 6.7-6.8). A weak BRET signal was observed for peptide-mediated GRK6 responses, which dropped down below baseline after the initial peak (albeit, these were very weak compared to that observed for GRK3). In addition, although both variants of GLP-1 and exendin-4 failed to induce an increase in the BRET response, a very weak reduction in the baseline response was observed compared to vehicle alone or compound 2 treatments. In contrast to the peptides and Compound 2, BETP displayed an increased BRET response that was sustained throughout the course of the experiment, suggesting a sustained interaction of the GLP-1R with GRK6. Furthermore, when either Compound 2 or BETP were co-added with peptide ligands, the profiles were the same as the profiles for the small molecules alone (Figure 6.7-6.8 A-D)

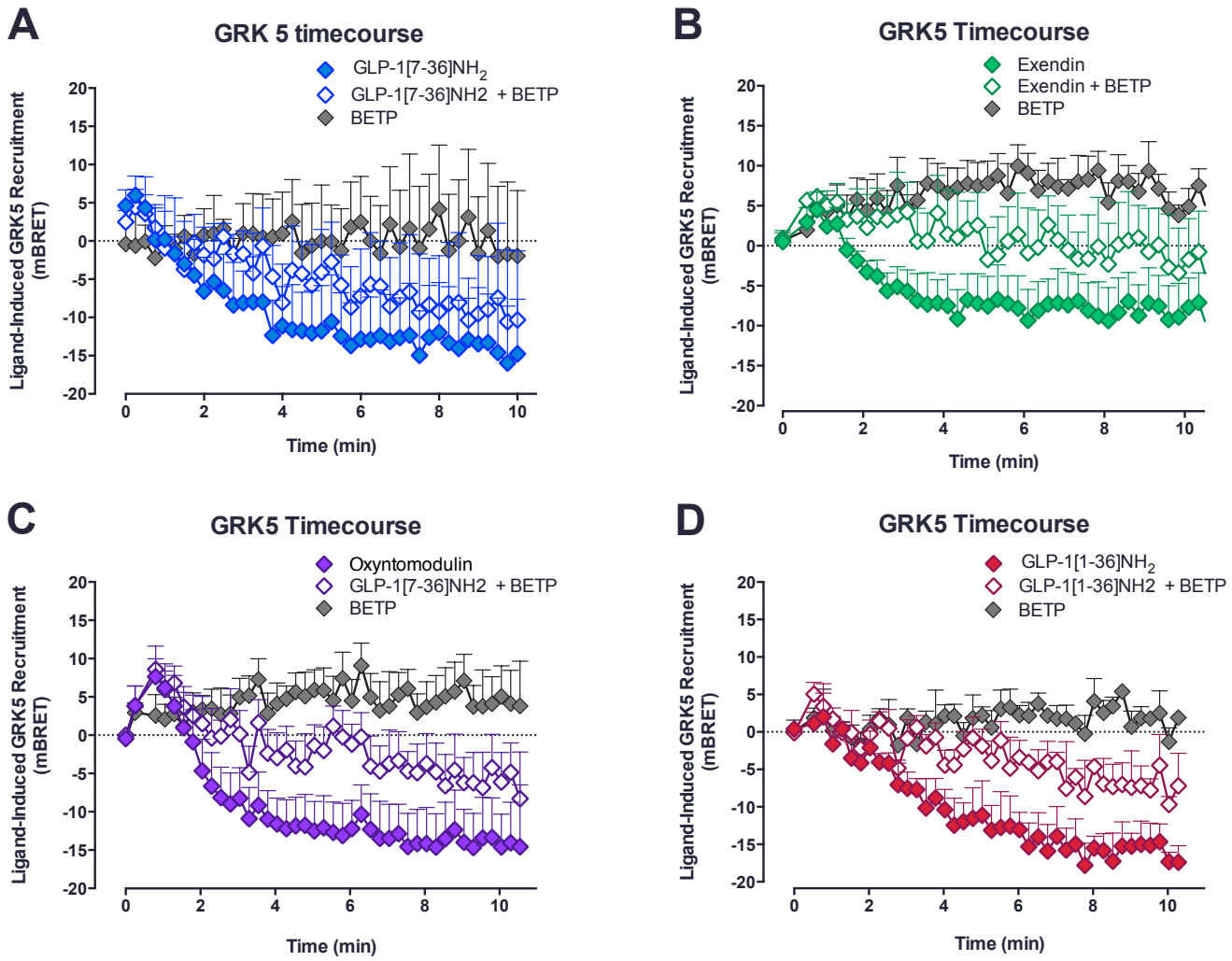

Figure 6.6 GRK5 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

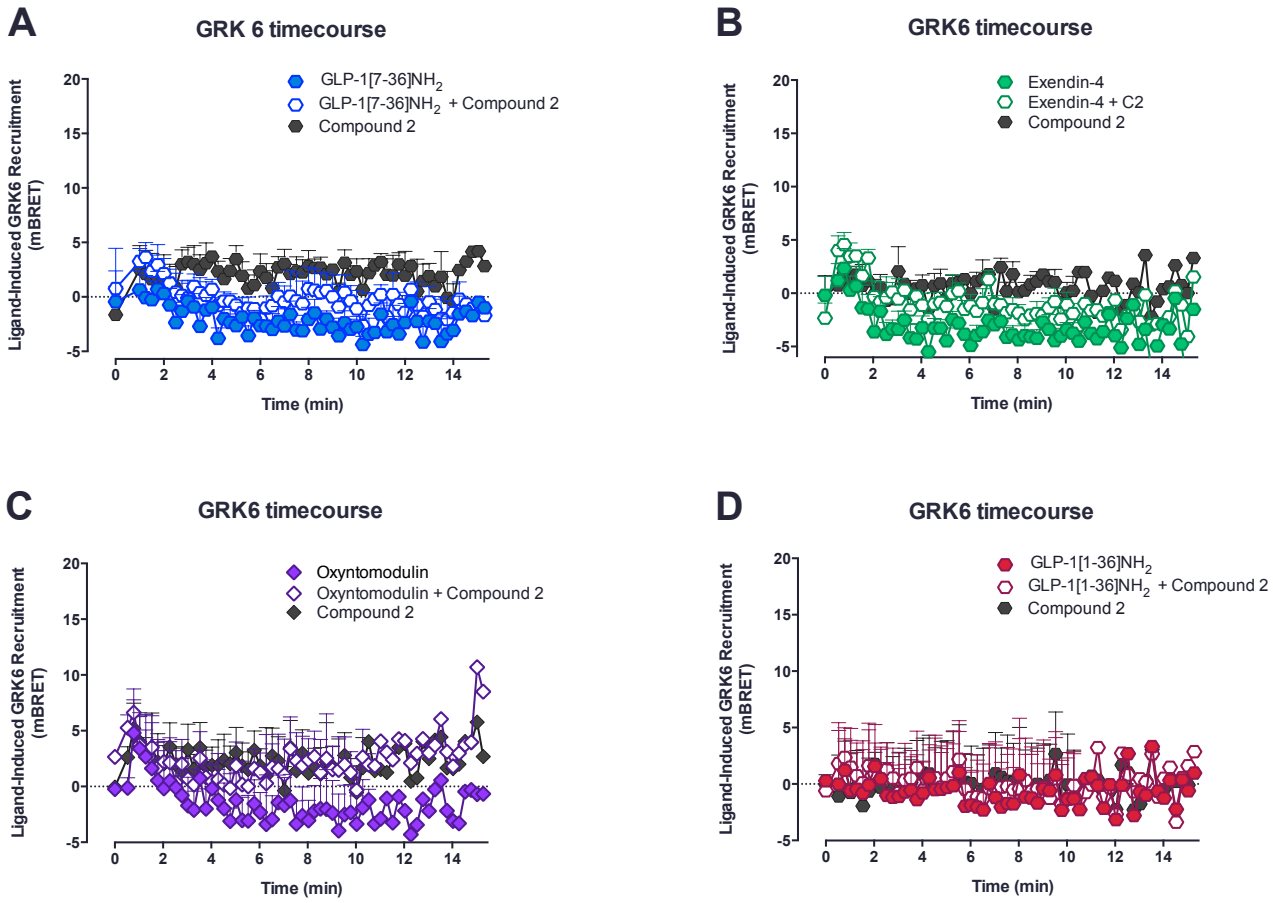

Figure 6.7. GRK6 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator Compound 2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

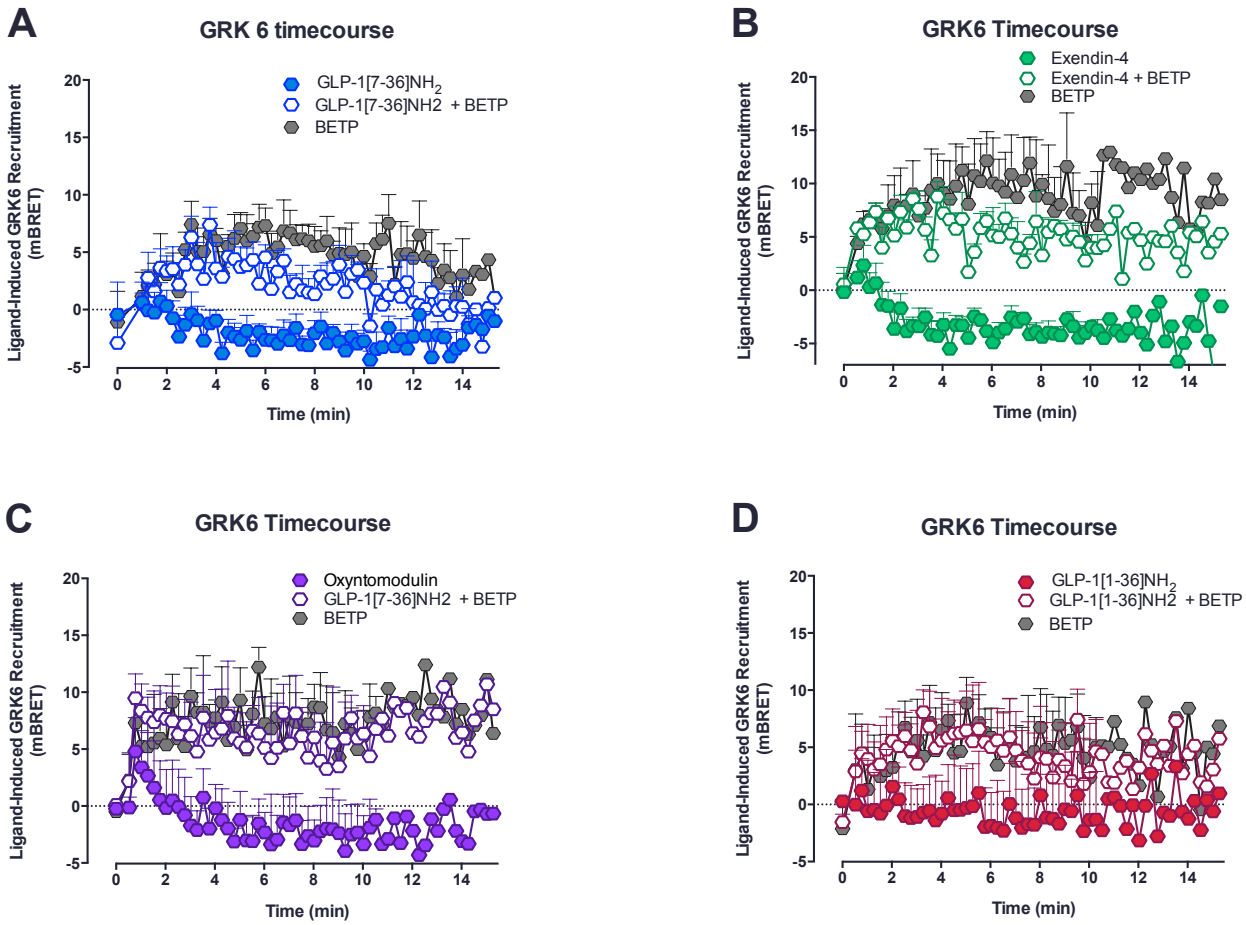

Figure 6.8 GRK6 recruitment time courses of GLP-1R agonists.

GLP-1(7-36)NH_2 (A), Exendin (B), Oxyntomodulin (C), and GLP-1(1-36) NH_2 (D), in the presence and absence of GLP-1R small molecule allosteric modulator BETP. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Boc5 (10 μM), TT15 (10 μM) and BMS21 (1 μM) displayed no change compared to vehicle alone in the BRET profile and therefore did not recruit detectable levels of GRK6 to the GLP-1R at the concentration of ligands tested (Table 6.2).

Distinct ligands can selectively recruit GRKs -2, -3, -5 and -6 to the GLP-1R in a concentration dependent manner.

Concentration response curves for GRK2, -3, -5, and -6 recruitment were generated at the peak response time point for each peptide ligand (Figure 6.9; Table 6.1), and for Compound 2 and BETP (Figure 6.10; Table 6.2). As no detectable GRK recruitment was observed for Boc5, TT15 or BMS21, these were excluded from the remainder of experiments.

The native peptide GLP-1(7-36)NH_2 was a strong agonist for recruitment of GRK2, -3, and -5. The most potent response was for GRK3 with a pEC_{50} of 8.1 ± 0.1, followed by GRK5 and GRK2 that had equivalent potency with a pEC_{50} of 7.4 ± 0.2 and 7.5 ± 0.3 respectively. Consistent with the timecourse, no recruitment of GRK6 was detected in response to GLP-1(7-36)NH_2 (Figure 6.9D; Table 6.1). Exendin-4 displayed a similar profile to that of GLP-1(7-36)NH_2, with highest potency for GRK3 (pEC_{50} 8.4 ± 0.2), followed by GRK2 (pEC_{50} 8.0 ± 0.3) and GRK5 (pEC_{50} 7.5 ± 0.2). Interestingly, unlike GLP-1(7-36)NH_2, exendin-4 was also able to recruit GRK6, albeit very weakly with a pEC_{50} of 7.4 ± 0.6 (Figure 6.9; Table 6.1). Oxyntomodulin also strongly recruited GRK2, GRK3 and GRK5 to the GLP-1R, however with lower potencies than GLP-1(7-36)NH_2 (Figure 6.9; Table. 6.1). In addition, for GRK5 recruitment, oxyntomodulin also displayed a significantly lower E_{max} than GLP-1(7-36)NH_2 (Figure 6.9; Table 6.1). There was no significant concentration response for oxyntomodulin-mediated recruitment of GRK6 (Fig 6.9; Table. 6.1). In agreement with the timecourse

experiments, no response to GLP-1(1-36)NH_2 was observed for mediated GRK2, 5 or 6 recruitment at 2.5 mins

Table 5.1

Differential effects of peptide agonists of the human GLP-1R in GRK2, -3, 5 and 6 recruitment in FlpInCHO cells that stably express the GLP-1R-Rluc8 and GRK-venus. pEC_{50} values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. All values are mean ± S.E.M. of three to five independent experiments, conducted in duplicate.

	GRK2		**GRK3**		**GRK5**		**GRK6**	
Ligand	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	$E_{max(mBRET)}$
GLP-1(7-36)NH_2	7.5± 0.3	96.98 ± 13.73	8.1± 0.1	98.8 ± 4.4	7.4± 0.2	104.4 ± 11.6	-	-
Exendin	8.0±0.3	82.37± 14.11	8.4± 0.2	87.51± 5.5	7.5± 0.2	93.7 ± 9.3	7.4 ± 0.6	2.9 ± .8
Oxyntomodulin	6.8±0.4	118.5± 32.00	7.2± 0.2	85.5± 7.1	6.8 ± 0.3	77.6 ± 13.3	-	-
GLP-1(1-36)NH_2	8.7±1.7	0.6± 9.6	5.36± 0.7	36.6 ± 2.8	7.4± 2.4	2.4 ± 7.4	-	-

Table 5.2

Differential effects of peptide/small molecule agonists of the human GLP-1R in β-arrestin1 and β-arrestin2 recruitment in FlpInCHO cells stably expressing the human GLP-1R. pEC_{50} values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. All values are mean ± S.E.M. of three to five independent experiments, conducted in duplicate, (ND = no detectable response).

	GRK2		**GRK3**		**GRK5**		**GRK6**	
Ligand	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	$E_{max(mBRET)}$
GLP-1(7-36)NH_2	7.5± 0.3	97.0 ± 13.7	8.1± 0.1	99.4 ± 2.9	7.4± 0.2	103.0 ± 10.1	-	-
Compound 2	5.2±0.3	133.7 ± 32.0	4.7± 0.5	44.3± 22.3	5.2± 0.3	80.7 ± 15.6	5.3± 0.3	5.3 ± 0.9
BETP	5.4±0.4	88.8 ± 29.6	5.4± 0.1	44.6 ± 4.4	5.7 ± 2.5	8.3± 2.5	4.7 ± 0.5	9.7 ± 5,5
Boc5	ND	ND	ND	ND	ND	ND	ND	ND
TT15	ND	ND	ND	ND	ND	ND	ND	ND
BMS21	ND	ND	ND	ND	ND	ND	ND	ND

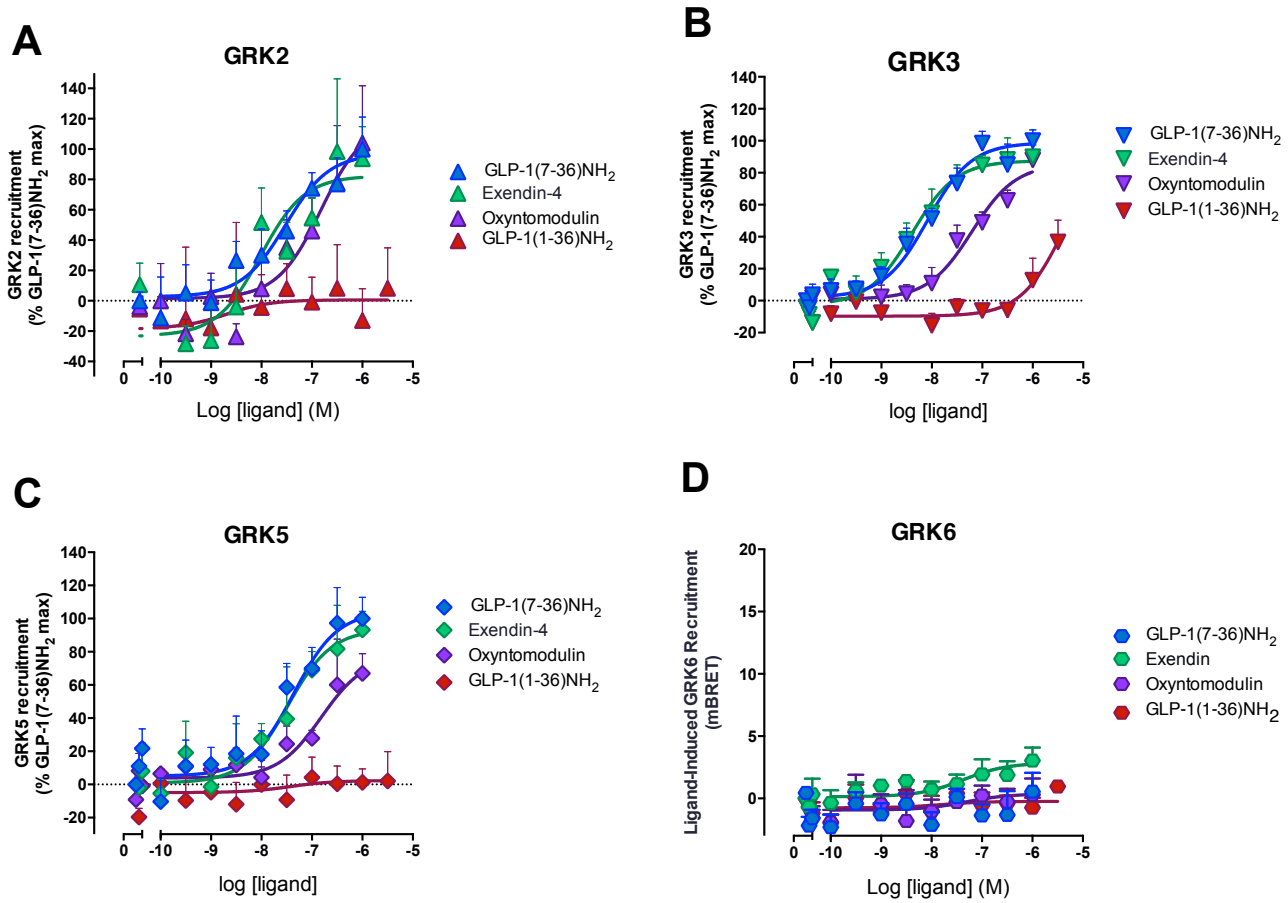

Figure 6.9 Peptide-induced recruitment of GRK2, -3, -5 and -6 to the GLP-1R.

Concentration response curves for GRK2 (A), GRK3 (B), GRK5 (C), and GRK6 (D) recruitment to the GLP-1R for GLP-1(7-36)NH_2, Exendin, Oxyntomodulin and GLP-1(1-36)NH_2. All data were analysed using a three-parameter logistic equation. Graphs A-C, are normalized to the response elicited by GLP-1(7-36)NH_2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

post ligand addition. Interestingly however, GLP-1(1-36)NH_2 was able to recruit GRK3 although within the ligand concentration tested, a full concentration response curve could not be generated (Figure 6.9B; Table 6.1).

Interestingly, both Compound 2 and BETP displayed agonism for recruitment of GRK2 with similar E_{max} values as GLP-1(7-36)NH_2, however with lower potencies (pEC_{50} of 5.2 ± 0.3 and 5.4 ± 0.4 respectively), that can be accounted for by their much lower affinities for the GLP-1R (Figure 6.10 A; Table 6.2). Furthermore, Compound 2 and BETP were weak partial agonists for GRK3 recruitment, with BETP showing a higher potency than that of Compound 2, (Figure 6.10 B; Table 6.2). With exception of BETP-mediated recruitment of GRK5, small molecules also displayed agonism for GRK5 and GRK6 at 2.5 mins (but this was only observed at the highest concentration tested and therefore full concentration response curves could not be achieved) (Figure 6.10 D; Table 6.2).

As Compound 2 and BETP were able to recruit almost all GRKs with weak responses, we also assessed Compound 2 and BETP at an M1 mAchR-Rluc8 GRK2-venus cell line to confirm the specificity of the GRK recruitment observed at these high concentrations of BETP and Compound 2. This revealed no detectable recruitment of GRK2 to the unrelated M1 mAchR at high concentrations of BETP and Compound 2.

Compound 2 and BETP selectively modulate peptide-mediated recruitment of GRKs to the GLP-1R

Compound 2 displayed a degree of positive cooperativity for GRK2 recruitment when coadded with all three peptides oxyntomodulin ($\alpha\beta$=19.1), exendin-4 ($\alpha\beta$=36.3) and GLP-1(7-36)NH_2 ($\alpha\beta$=29.5), although oxyntomodulin did not reach statistical significance.

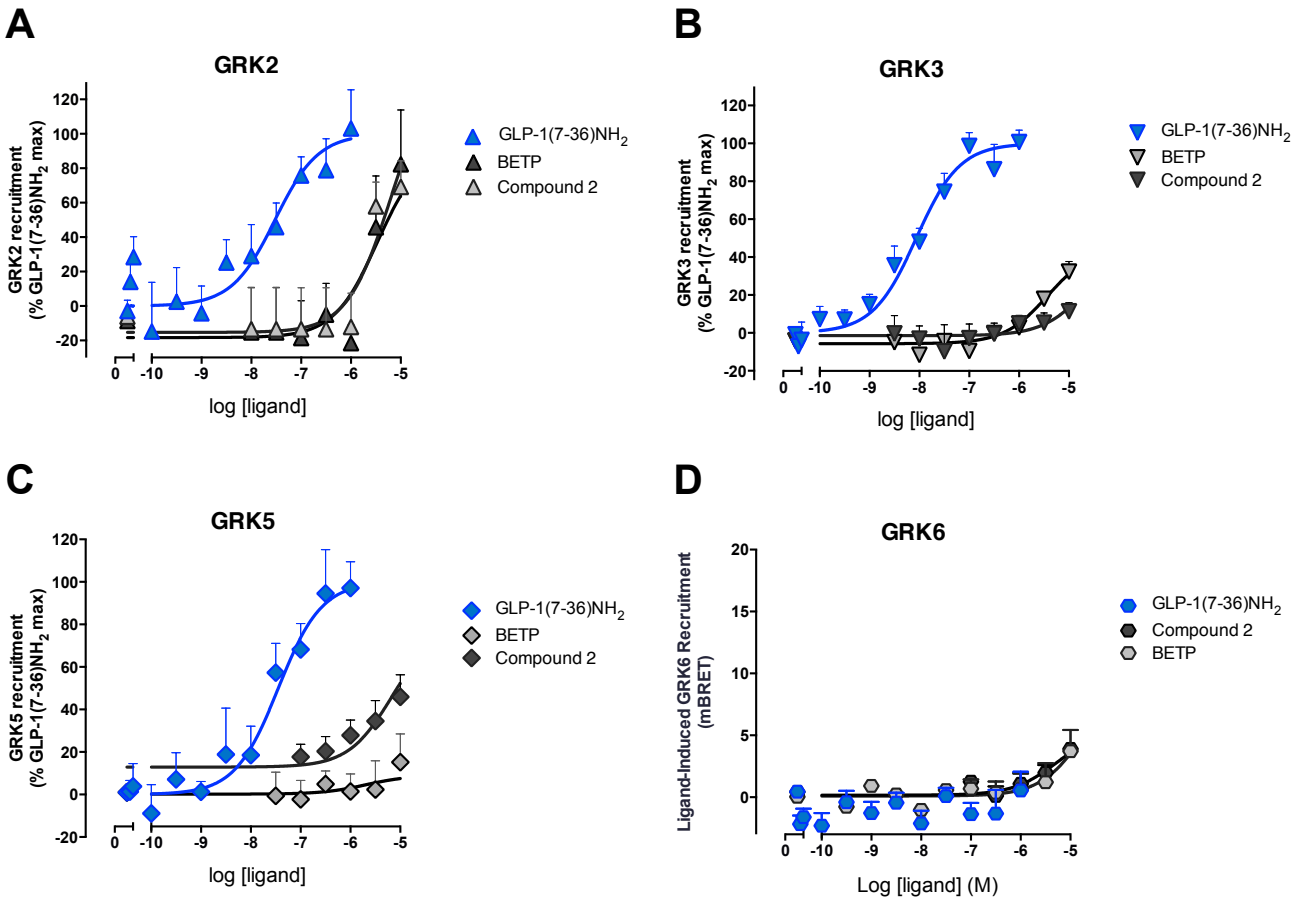

Figure 6.10. Small molecule-induced recruitment of GRKs to the GLP-1 receptor.

Concentration response curves for GRK2 (A), GRK3 (B), GRK5 (C), and GRK6 (D) to the GLP-1 receptor for GLP-1(7-36)NH_2, Compound 2 and BETP. All data were analysed using a three-parameter logistic equation. Graphs A-C, are normalized to the response elicited by GLP-1(7-36)NH_2. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

(Figure 6.11 A-C; Table 6.3). However, for GRK3 recruitment, Compound 2 was a much weaker positive modulator and the rank order of positive cooperativity for the peptides was different compared to GRK2. Oxyntomodulin displayed the most significant modulation with a combined αβ cooperativity of 27.5 ($p < 0.05$) followed by GLP-1(7-36)NH_2 (αβ=12.9, $p < 0.05$) with only modest modulation of exendin-4 (αβ=5.03) (Figure 6.12 A-C; Table 6.3).

Similar to Compound 2, BETP displayed significant positive cooperativity with exendin-4 (αβ=46.8, $p< 0.05$) for GRK2 recruitment and to a lesser extent oxyntomodulin (αβ=21.4), followed by GLP-1(7-36)NH2 (αβ=8.5) although the latter two peptides were not statistically significant (Figure 6.15 A-C; Table. 6.3). In contrast to Compound 2, BETP did not significantly modulate GRK3 recruitment for any peptide (Figure 6.12 A-C; Table 6.3).

Neither BETP or Compound 2 were able to modulate GRK2 or 3 recruitment in the presence of GLP-1(1-36)NH_2 (Figure 6.11 D; 6.12 D, 6.13 D; 6.14 D; Table 6.2). In addition, no modulation could be detected for GRK5 or GRK6 in the presence of the any combination of small molecule and peptide tested (Figure 6.15-6.18, Table 6.3).

The role of GRKs on β-arrestin recruitment to the GLP-1R

The role of GRK2, -3, -5 and -6 for β-arrestin1 and β-arrestin2 recruitment to the GLP-1R was assessed by measuring ligand-mediated β-arrestin1 and β-arrestin2 recruitment in the presence of dominant negative constructs of each GRK. FlpInCHO cell lines stably expressing containing GLP-1R-Rluc8 and either βarrestin1- or βarrestin2-Venu were transiently transfected with dominant negative GRK pcDNA3.1 vectors (GRK 2,-3,-5, and -6). These experiments were performed in the GLP-1R-Rluc8, β-arrestin-Venus ChoFlpIn cell lines presented in Chapter 5 (Willard, Wootten, et al. 2012b; Savage et al. 2013).

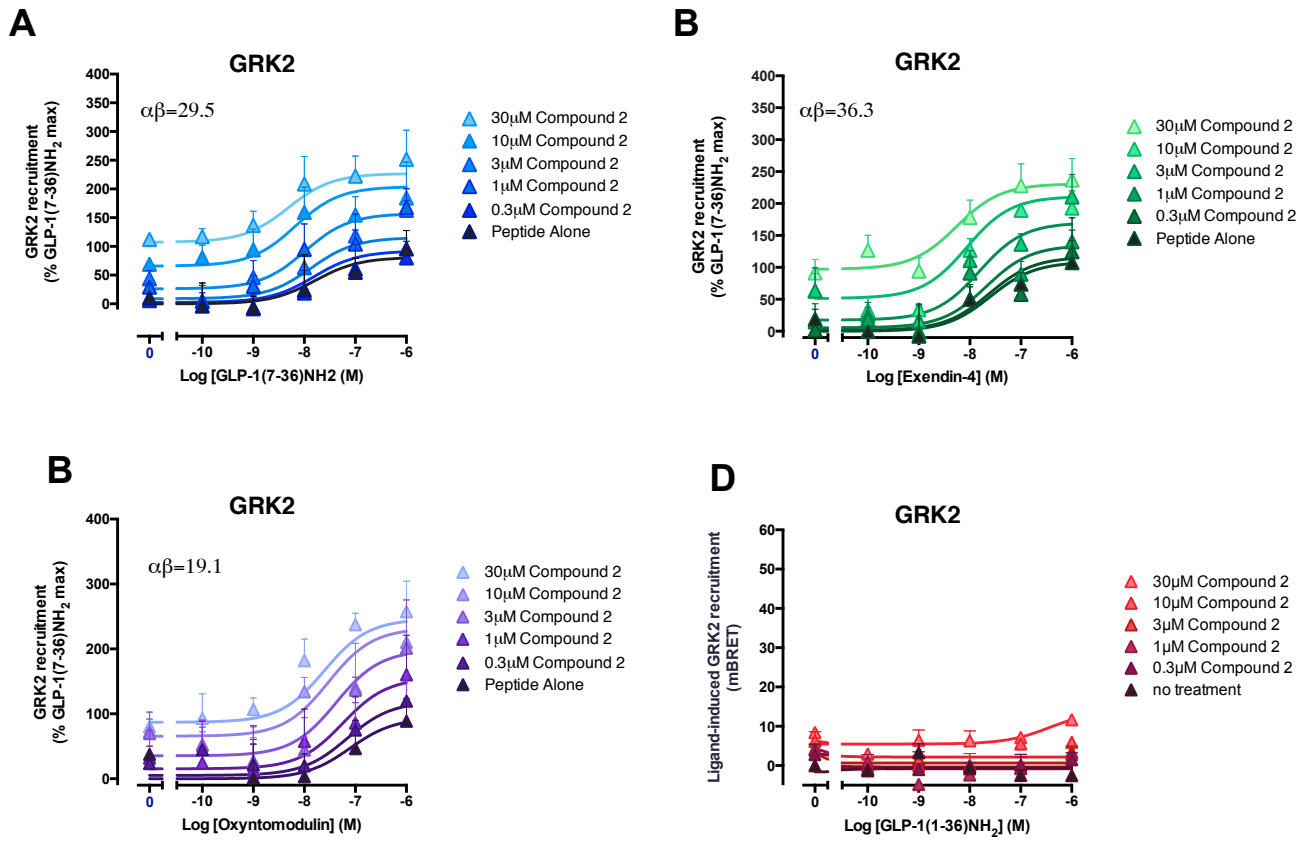

Figure 6.11 Compound 2 potentiates GLP-1R-mediated recruitment of GRK2 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 (D), in the absence and presence of increasing concentrations of Compound 2 for GRK2 recruitment. Compound 2 did not potentiate GLP-1R-mediated recruitment GRK2 by GLP-1(1-36)NH_2. Curves A-B represent the best global fit of an operational model of allosterism (eq. 5), normalized to the response elicited by peptide alone. Graph D was analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

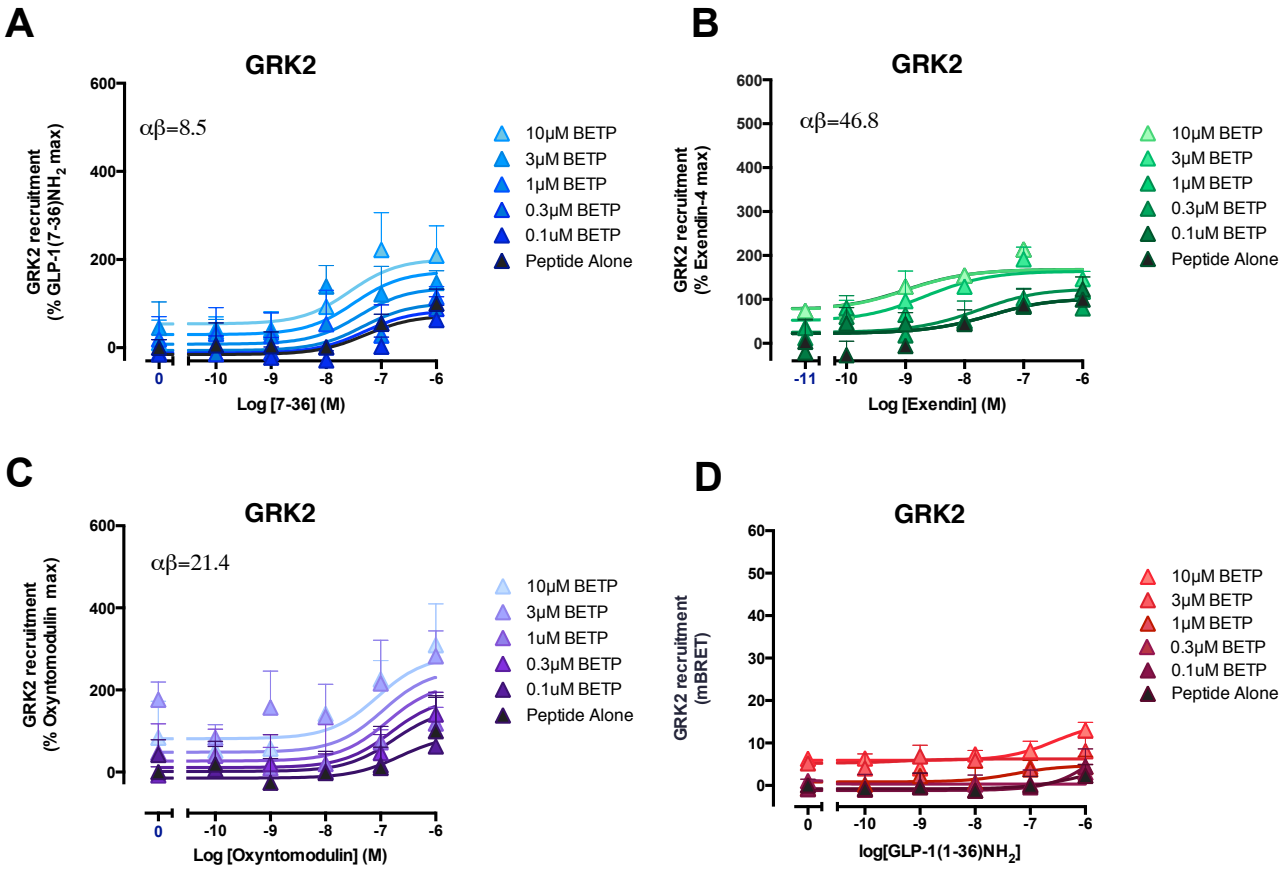

Figure 6.12 BETP potentiates GLP-1R-mediated recruitment of GRK2 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 (D), in the absence and presence of increasing concentrations of BETP for GRK2 recruitment. BETP did not potentiate GLP-1R-mediated recruitment GRK2 by GLP-1(1-36)NH_2. Curves (A-C) represent the best global fit of an operational model of allosterism (eq. 5). Where data from (D) was analyzed using a three-parameter logistic equation All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

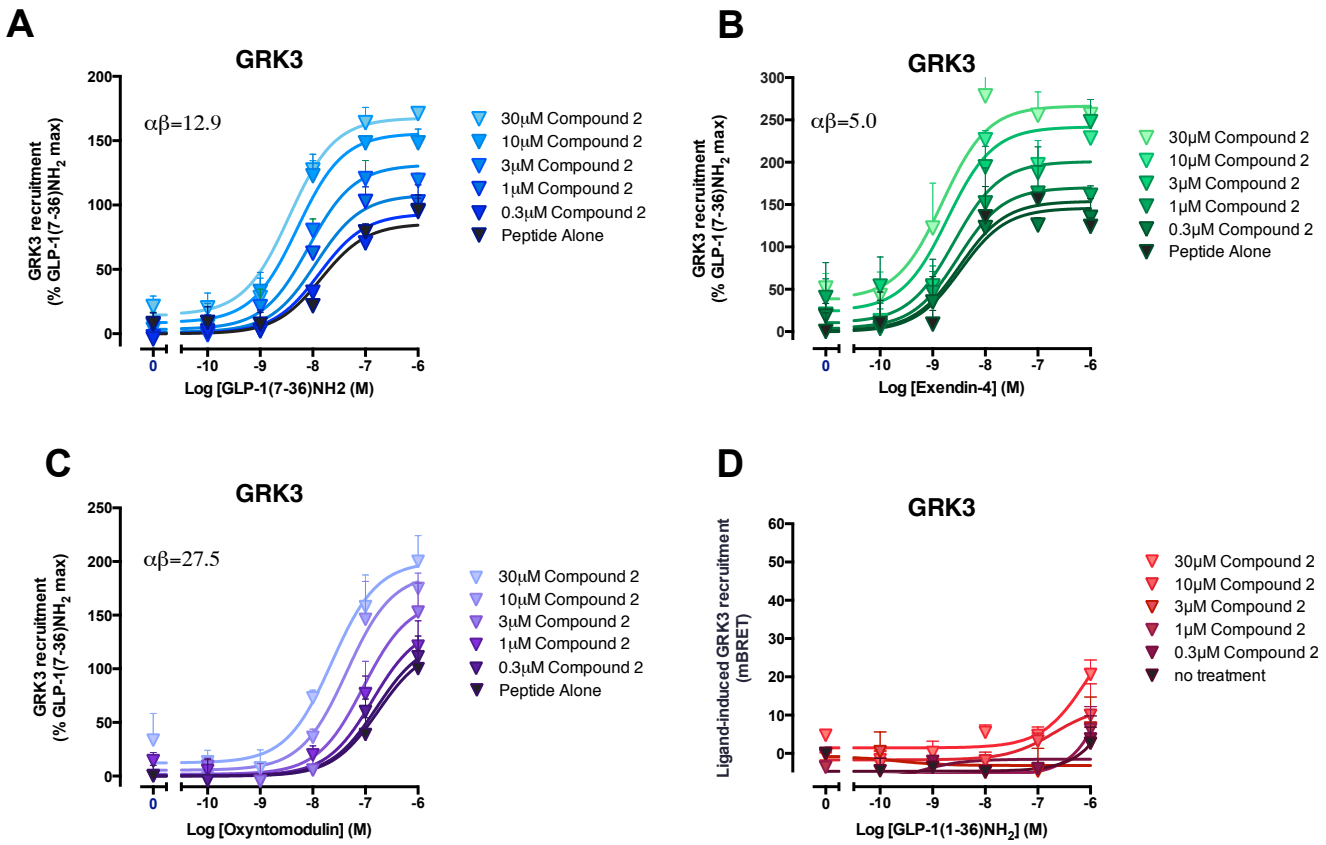

Figure 6.13 Compound 2 potentiates GLP-1R-mediated recruitment of GRK3 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 (D) in the absence and presence of increasing concentrations of Compound 2 for GRK3 recruitment. Compound 2 did not potentiate GLP-1R-mediated recruitment GRK3 by GLP-1(1-36)NH_2. Curves A-B represent the best global fit of an operational model of allosterism (eq. 5), normalized to the response elicited by peptide alone. Graph D was analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

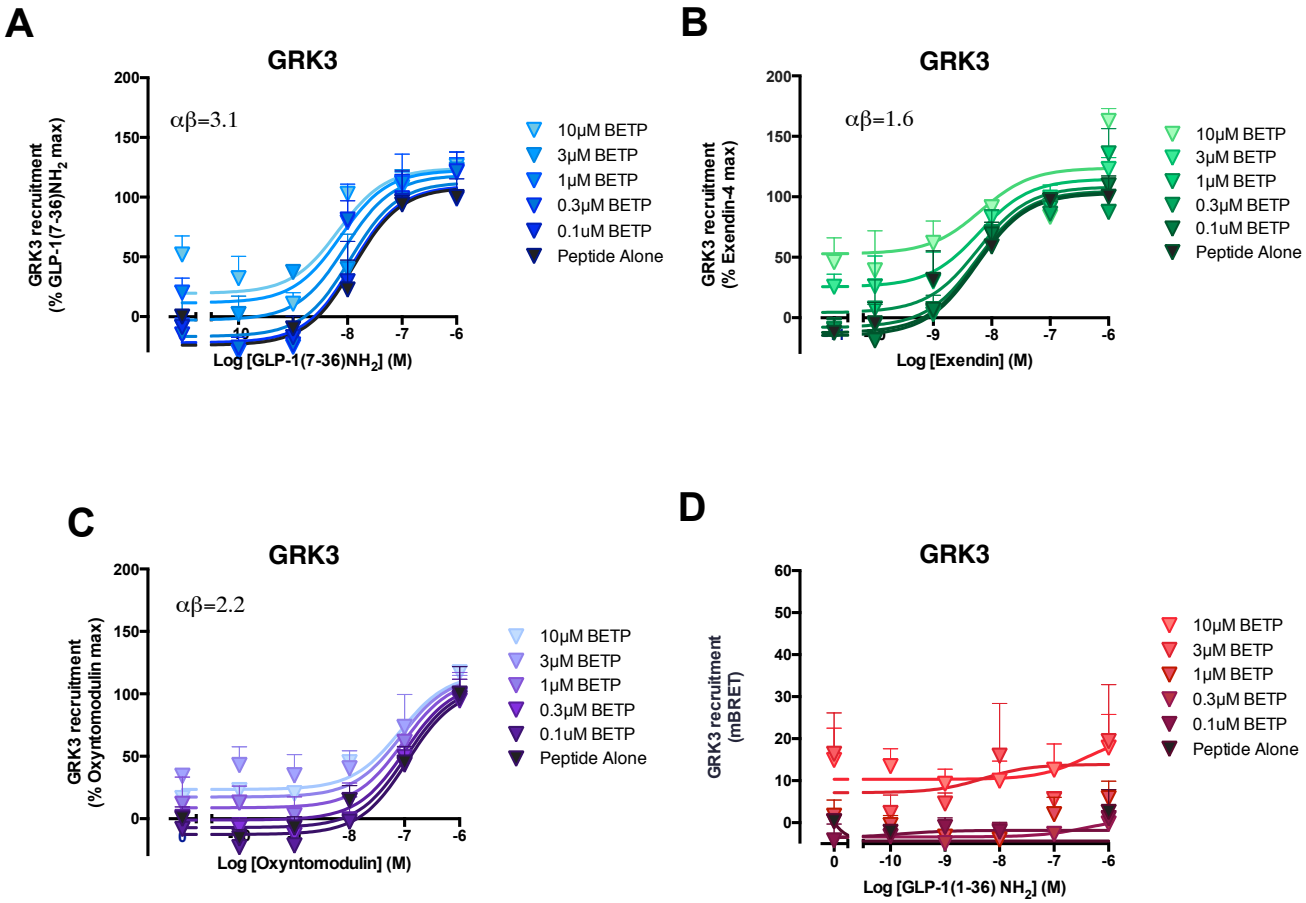

Figure 6.14 BETP potentiates GLP-1R-mediated recruitment of GRK3 by peptide ligands. Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 (D) in the absence and presence of increasing concentrations of BETP for GRK3 recruitment. BETP did not potentiate GLP-1R-mediated recruitment GRK3 by GLP-1(1-36)NH_2. Curves (A-C) represent the best global fit of an operational model of allosterism (eq. 5). Where data from (D) was analyzed using a three-parameter logistic equation All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

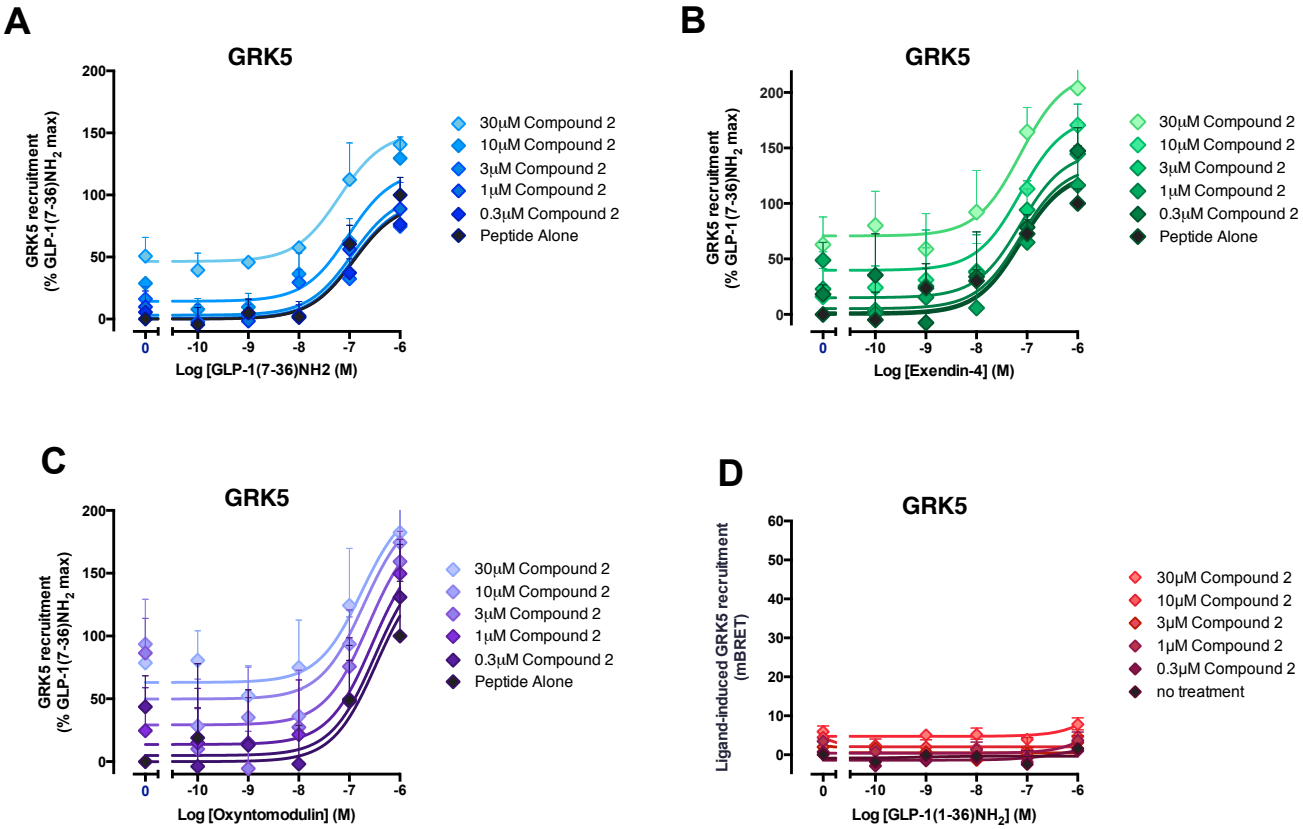

Figure 6.15 Compound 2 does not significantly alter GLP-1R-mediated recruitment of GRK5 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of Compound 2 for GRK5 recruitment. Compound 2 did not potentiate GLP-1R-mediated recruitment of GRK5 by GLP-1(1-36)NH_2. Curves A-B represent the best global fit of an operational model of allosterism (eq. 5), normalized to the response elicited by peptide alone. Graph D was analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

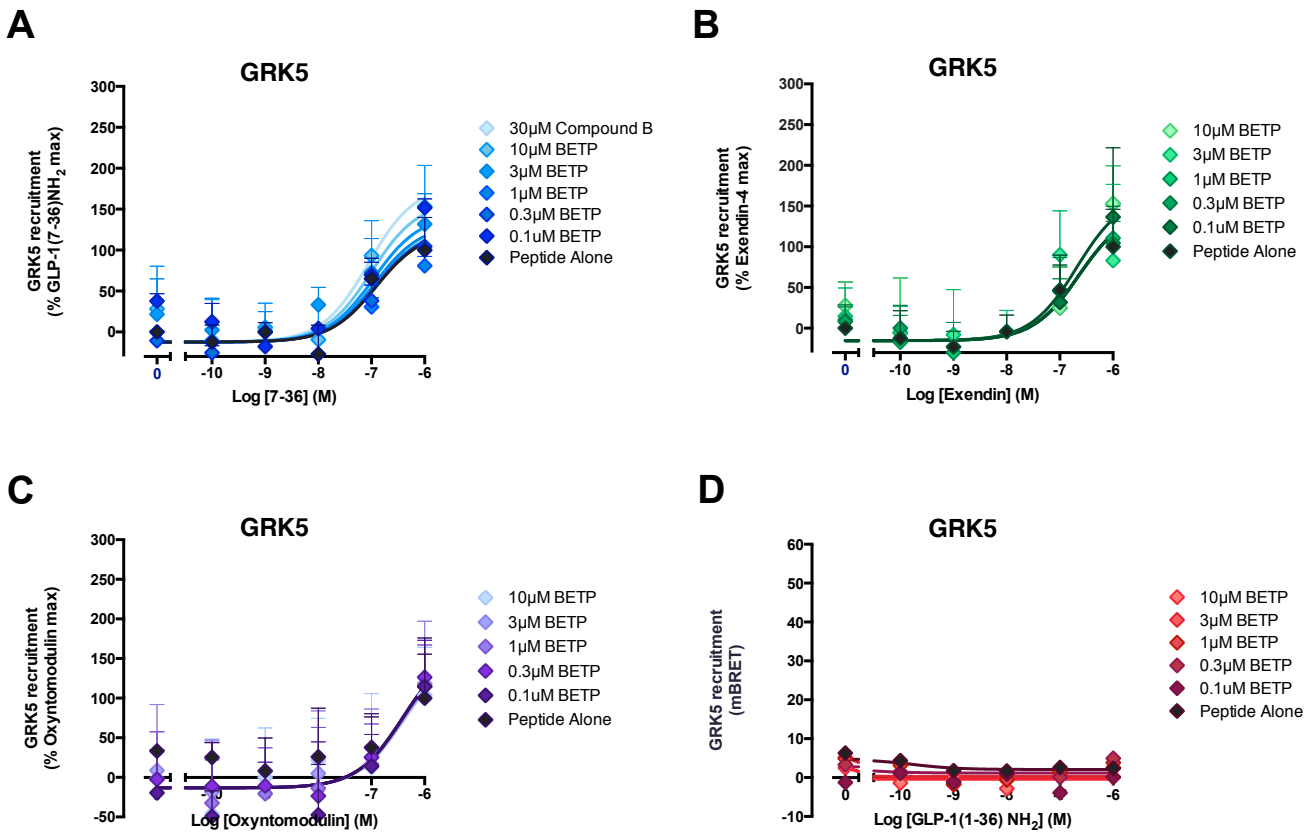

Figure 6.16 BETP does not alter GLP-1R-mediated recruitment of GRK5 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of BETP for GRK5 recruitment. BETP did not potentiate GLP-1R-mediated recruitment of GRK5 by GLP-1(1-36)NH_2. Curves (A-C) represent the best global fit of an operational model of allosterism (eq. 5). Where data from (D) was analyzed using a three-parameter logistic equation All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

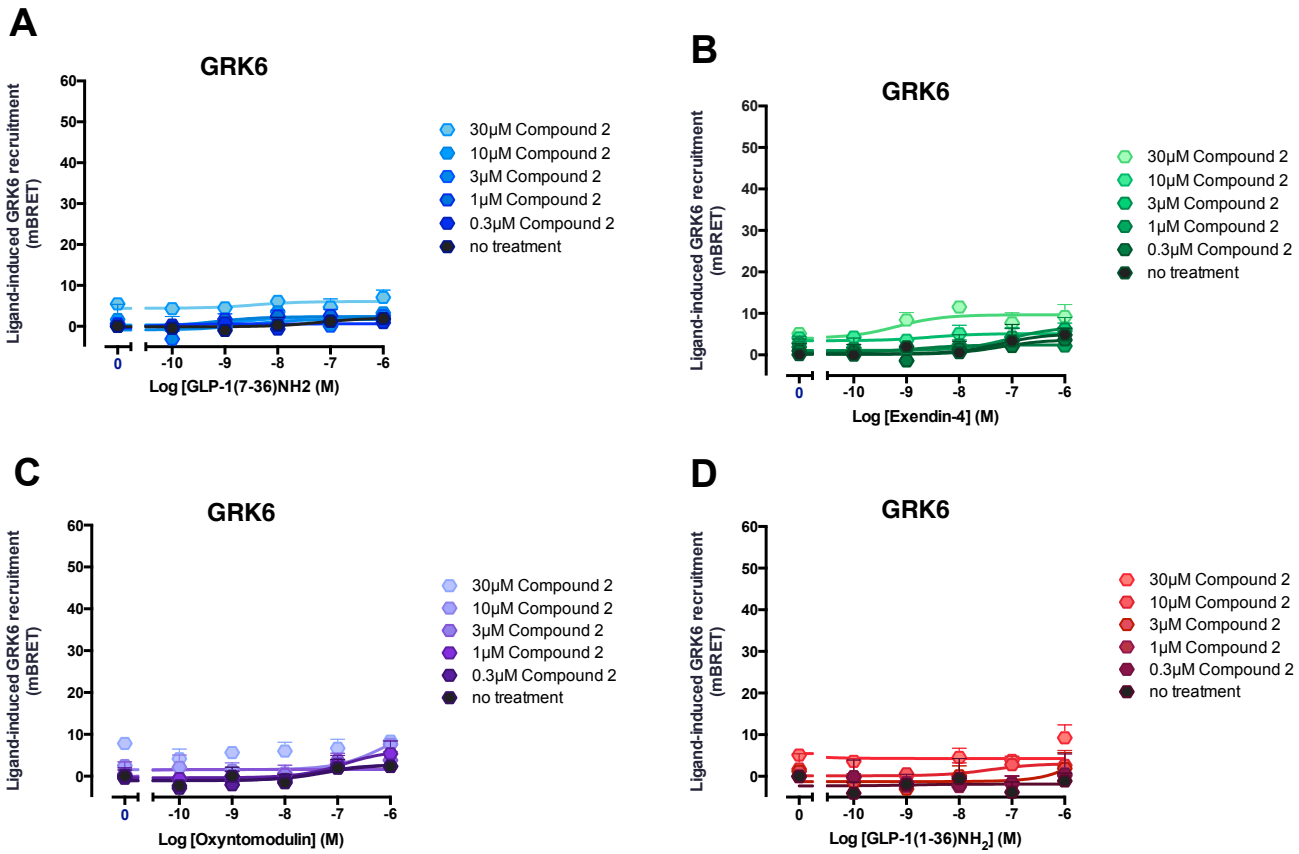

Figure 6.17 Compound 2 does not alter GLP-1R-mediated recruitment of GRK6 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of Compound 2 for GRK6 recruitment. Compound 2 did not potentiate GLP-1R-mediated recruitment of GRK6 by any of the peptides. Data were analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

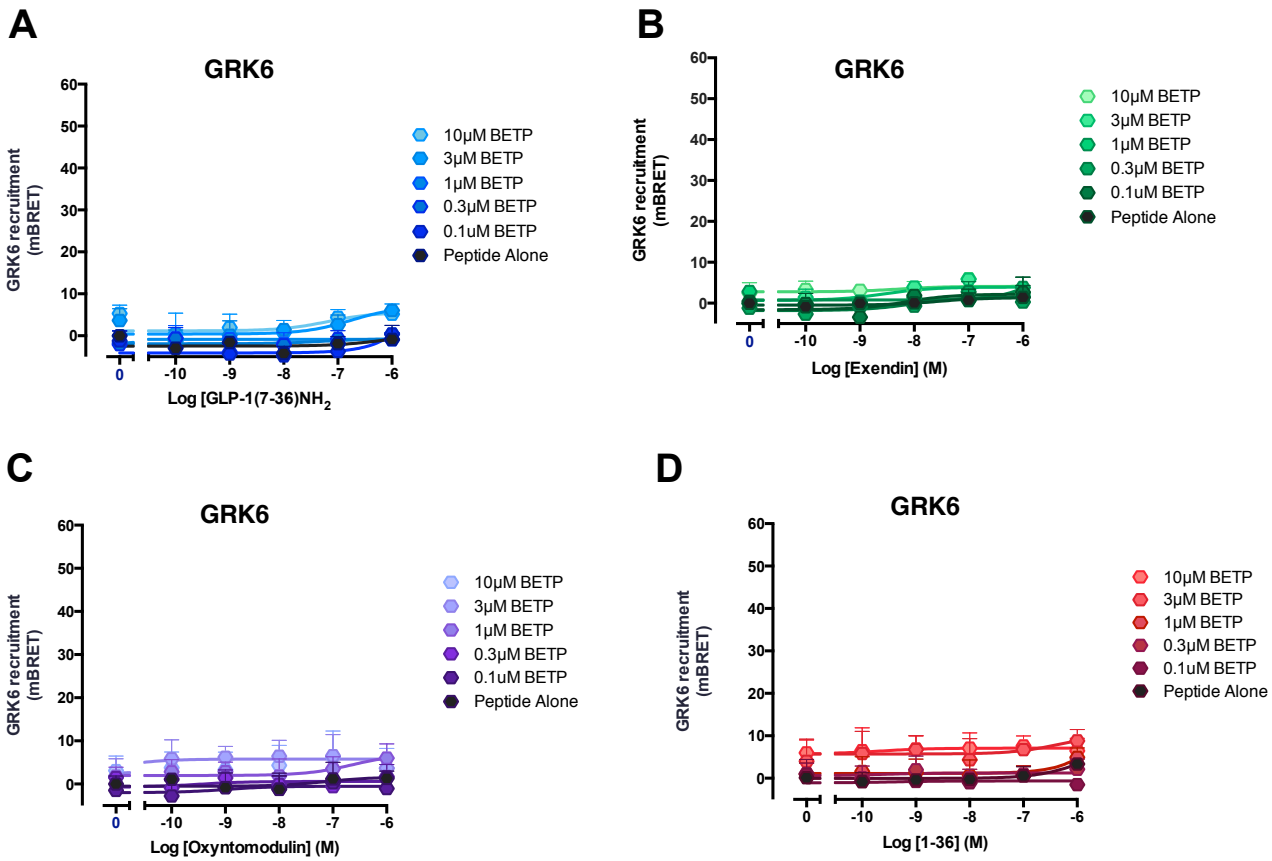

Figure 6.18 BETP does not alter GLP-1R-mediated recruitment of GRK6 by peptide ligands.

Concentration response curves were generated for GLP-1(7–36)NH_2 (A), exendin-4 (B), oxyntomodulin (C) and GLP-1(1-36)NH_2 in the absence and presence of increasing concentrations of BETP for GRK6 recruitment. BETP did not potentiate GLP-1R-mediated recruitment of GRK6 by any of the peptides. Data were analyzed using a three-parameter logistic equation. All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

Table 6.3. Functional cooperativity estimates for the interaction between BETP or Compound 2 and GLP-1R peptide ligands.
Data derived from analysis of interaction dose-response curves with an operational model of allosterism as defined in equation 4. pKb values are the negative logarithms for the functional affinity of the allosteric ligands, log αβ represent the composite cooperativity between the allosteric ligand and the orthosteric peptide ligand. Antilogarithms are shown in parentheses. Values represent the mean ± S.E.M of four to six independent experiments performed in duplicate. Data were analysed with one-way analysis of variance and Dunnett's post test ($^{*}p < 0.05$).

Pathway	Allosteric Ligand	pKb	Log αβ (αβ)			
			Exendin-4	GLP-1(7-36)NH_2	Oxyntomodulin	GLP-1(1-36)NH_2
β-Arr1	BETP	5.42 ± 0.17	-0.05 ± 0.04 (0.89)	-0.01 ± 0.02 (1.0)	0.40 ± 0.17 (2.5)	-
	Cpd2	5.27 ± 0.18	0.72 ± 0.18 (5.2)*	1.07 ± 0.19 (12)*	1.05 ± 0.14 (11)*	-
β-Arr2	BETP	5.38 ± 0.16	0.18 ± 0.26 (1.5)	0.67 ± 0.18 (4.7)	0.54 ± 0.19 (3.5)	-
	Cpd2	5.30 ± 0.19	0.69 ± 0.20 (4.9)*	1.06 ± 0.13 (11)*	0.99 ± 0.19 (10)*	-
GRK2	BETP		1.67±0.48 (46.8)*	0.93±0.45 (8.5)	1.33±0.54 (21.4)	-
	Cpd2		1.56±0.23 (36.3)*	1.47±0.50 (29.5)*	1.28±0.33 (19.1)	-
GRK3	BETP		0.21±0.11 (1.62)	0.5±0.31 (3.1)	0.34±0.31 (2.2)	-
	Cpd2		0.70±0.34 (5.03)	1.11±0.14 (12.9)*	1.44±0.34 (27.5)**	-
GRK5	BETP		0.09±0.28(1.2)	0.29±0.12(2.0)	-0.07±0.12 (1.2)	-
	Cpd2		0.34±1.57(2.2)	0.93± 0.74 (8.5)	0.61±0.69 (4.1)	-
GRK6	BETP	-	-	-	-	-
	Cpd2	-	-	-	-	-

Interestingly the expression of dominant negative constructs of either GRK2, -3, -5 or -6 decreased the baseline BRET response (indicative of constitutive GRK interaction with the GLP-1R) for all four GRKs, however this was to different extents. This was most evident for the GRK3 dominant negative, whereby each baseline was significantly lower than control in every experimental condition $p < 0.05$. For GRK3 dominant negative there was a trend towards lower baseline compared to control. For GRK2, GRK5 and GRK6 dom/neg constructs some conditions were statistically significant.

Analysis of concentration response curves for GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin revealed that the GRK2 dominant negative reduced β-arrestin1 and β-arrestin2 recruitment to the greatest extent across all peptides, followed by GRK6 and GRK5, with the least effect on GRK3 (Figure 6.19-6.22; Table 6.4). Furthermore, GRK2 dominant negative had a greater effect on GLP-1(7-36)NH_2-mediated recruitment of β-arrestin1 and β-arrestin2 compared to other peptides, reducing the response to 25 % and 22 % of peptide response from the mock transfection, respectively (Figures 6.19 A; 6.20 A, Table 6.4). In the presence of GRK2 dominant negative, exendin-mediated recruitment of both β-arrestin1 and β-arrestin2 was reduced to 37 % and 32 % of mock transfection respectively while oxyntomodulin was least affected (40 % and 54 %) (Figures 6.19 B-C; 6.20 B-C, Table 6.4). GRK3 dominant negative had little effect on β-arrestin1 and β-arrestin2 recruitment for any peptide, (Figures 6.19 F; 6.20 F, Table 6.4), however the results are not surprising given that CHO cells do not appear to express GRK3, as reported by (Horie & Insel 2000).

Interestingly, β-arrestin1 and β-arrestin2 recruitment was substantially inhibited in the presence of dominant negative GRK5 and GRK6 when the receptor was activated by either GLP-1(7-

36)NH_2 or exendin-4, and to a lesser extent for oxyntomodulin responses (Figure 6.21-6.22; Table 6.4). Overall these results reveal differential roles of distinct GRK subtypes in β-arrestin

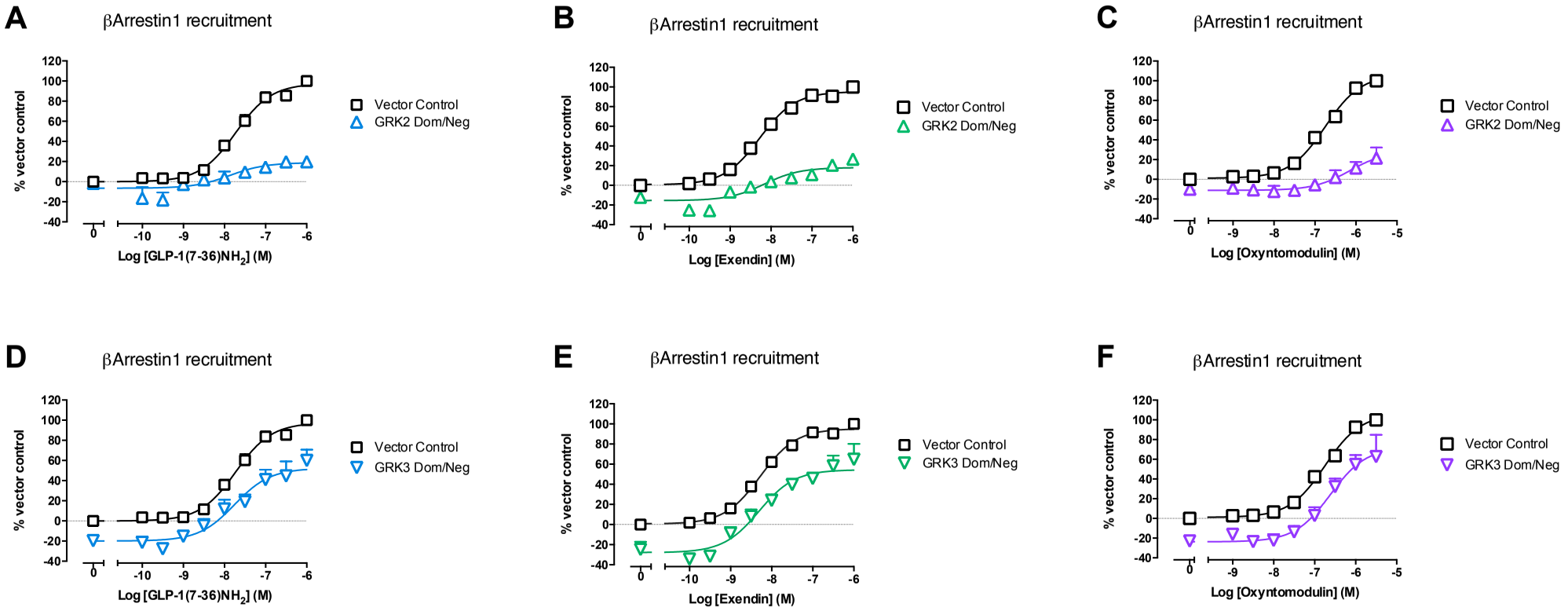

Figure 6.19 The effect of GRK2, and GRK3 dominant negative and overexpression on peptide-induced recruitment of β-arrestin1 to the GLP-1 receptor.

Concentration response curves for GRK-2-mediated (A, B, C) and GRK-3 mediated (D, E, F) β-arrestin1 recruitment in the presence of GLP-1(7-36)NH_2 (A, D), Exendin-4 (B, E) and Oxyntomodulin (C, F). Measurements were made in cells that were overexpressing ('vector control') or had knockdown ('dom/neg') of the indicated GRK. Measurements were made in cells that were overexpressing ('vector control') or had knockdown ('dom/neg') of the indicated GRKAll data were analyzed using a three-parameter logistic equation. Graphs are normalised to the response elicited by peptide. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

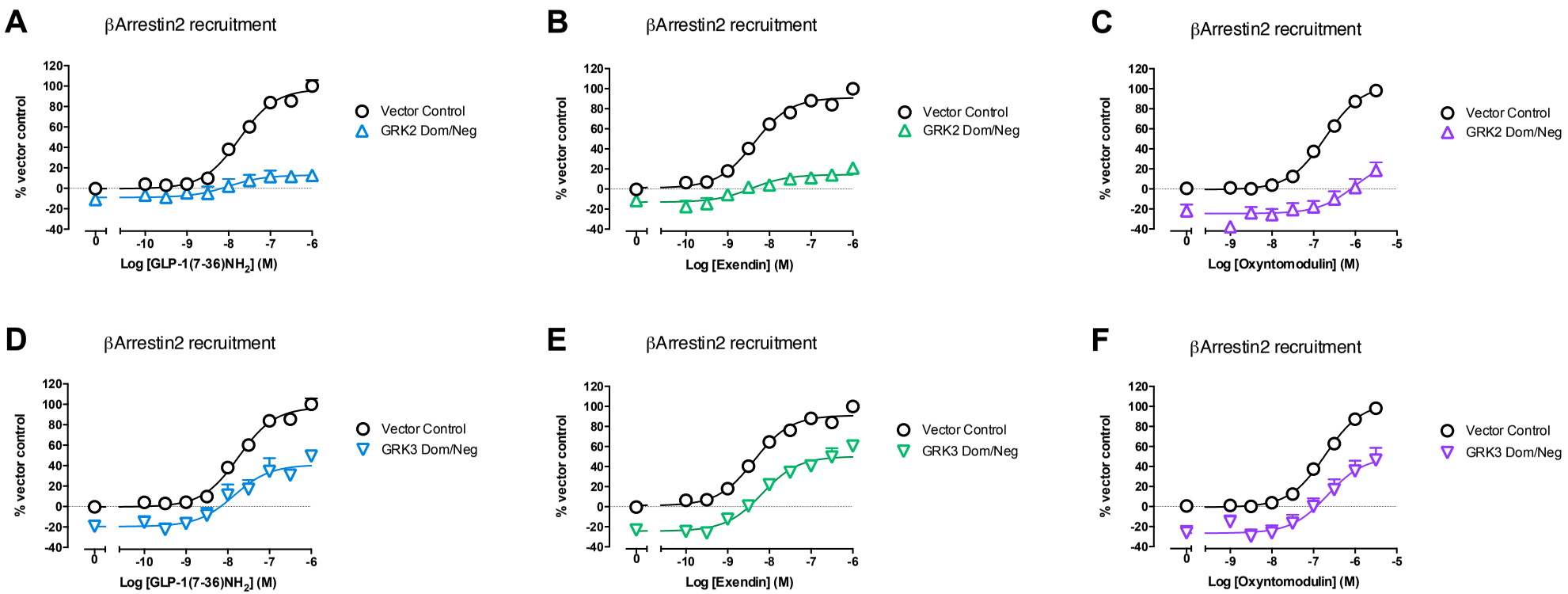

Figure 6.20 The effect of GRK2, and GRK3 dominant negative and overexpression on peptide-induced recruitment of β-arrestin2 to the GLP-1 receptor.

Concentration response curves for GRK-2-mediated (A, B, C) and GRK-3 mediated (D, E, F) β-arrestin2 recruitment in the presence of GLP-1(7-36)NH_2 (A, D), Exendin-4 (B, E) and Oxyntomodulin (C, F). Measurements were made in cells that were overexpressing ('vector control') or had knockdown ('dom/neg') of the indicated GRK. All data were analyzed using a three-parameter logistic equation. Graphs are normalized to the response elicited by peptide. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

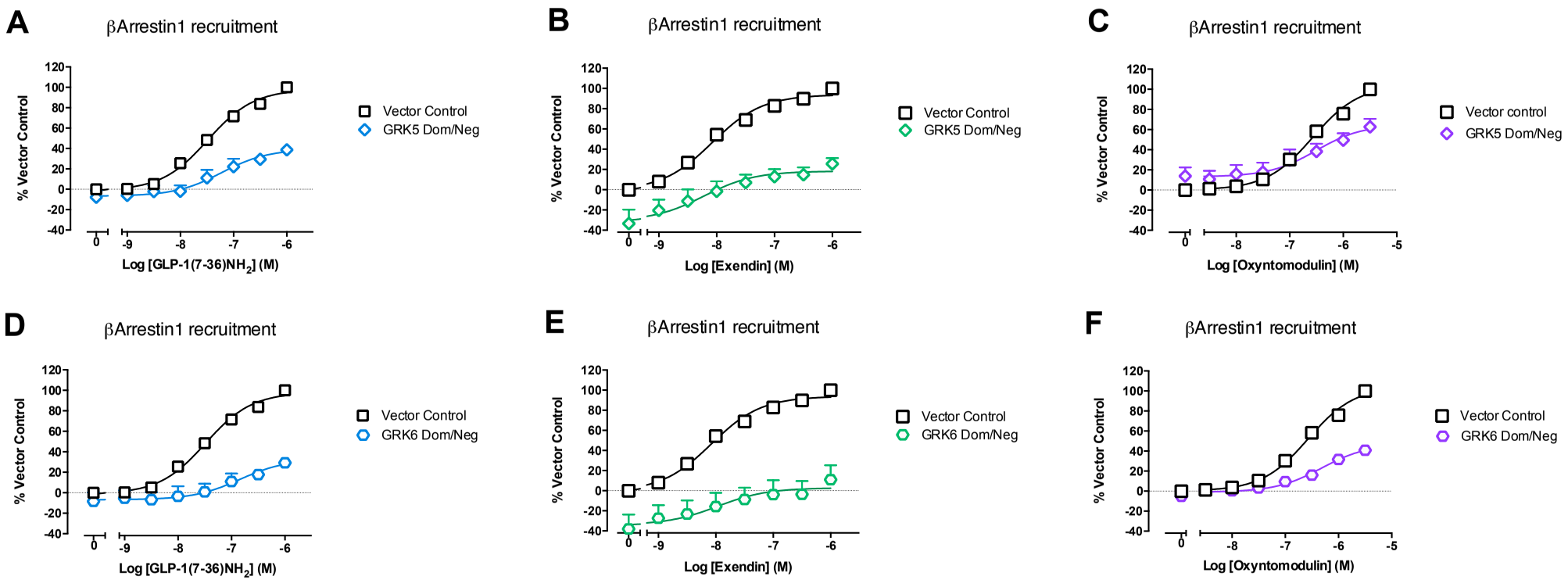

Figure 6.21 The effect of GRK5, and GRK6 dominant negative and overexpression on peptide-induced recruitment of β-arrestin1 to the GLP-1 receptor.

Concentration response curves for GRK-5-mediated (A, B, C) and GRK-6 mediated (D, E, F) β-arrestin1 recruitment in the presence of GLP-1(7-36)NH_2 (A, D), Exendin-4 (B, E) and Oxyntomodulin (C, F). Measurements were made in cells that were overexpressing ('vector control') or had knockdown ('dom/neg') of the indicated GRK. All data were analyzed using a three-parameter logistic equation. Graphs are normalized to the response elicited by peptide. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

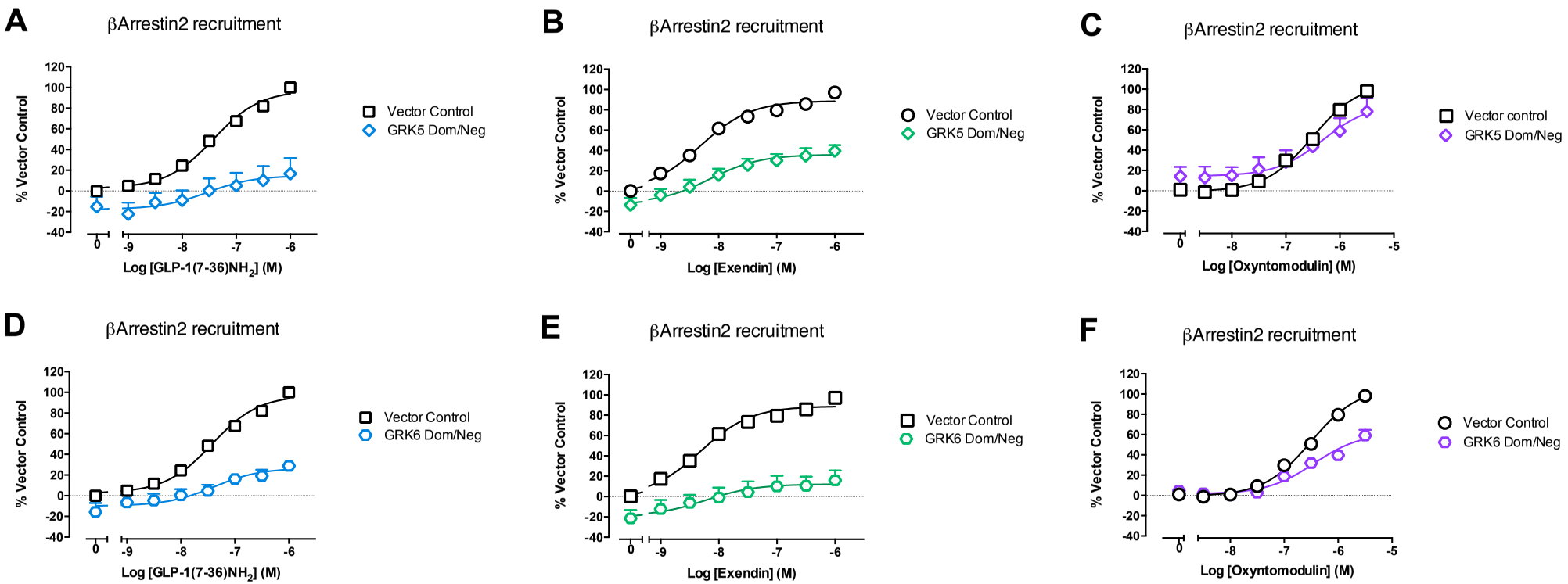

Figure 6.22 The effect of GRK5, and GRK6 dominant negative and overexpression on peptide-induced recruitment of β-arrestin2 to the GLP-1 receptor.

Concentration response curves for GRK-5-mediated (A, B, C) and GRK-6 mediated (D, E, F) β-arrestin2 recruitment in the presence of GLP-1(7-36)NH_2 (A, D), Exendin-4 (B, E) and Oxyntomodulin (C, F). Measurements were made in cells that were overexpressing ('vector control') or had knockdown ('dom/neg') of the indicated GRK. All data were analyzed using a three-parameter logistic equation. Graphs are normalized to the response elicited by peptide. All values are mean ± S.E.M. of three to four independent experiments conducted in duplicate.

Table 6.4. Differential effects of GRK dominant negatives with different peptides at the human GLP-1R on β-arrestin1 and β-arrestin2 recruitment in FlpInCHO cells. pEC_{50} values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. Range is established from response calculated from the top and bottom of concentration response curves. All values are mean ± S.E.M. of three to five independent experiments, conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with control agonist.

		β-Arrestin1				β-Arrestin2			
	Ligand	pEC_{50}	% Basal	% E_{max}	% Response (E_{max} – Basal)	pEC_{50}	Basal	E_{max}	% Response (E_{max} – Basal)
Control	GLP-1(7-36)NH_2	7.7±0.1	0±2	100.0±3	100	7.7±0.1	0±2	100±3	100
	Exendin-4	8.3±0.04	0±2	100±2	100	8.4±0.1	0±2	100±2	100
	Oxyntomodulin	6.7±0.1	0±2	100±4	100	6.7±0.1	0±2	100±3	100
GRK2 K220R	GLP-1(7-36)NH_2	7.9±0.3	-7±3	19±4	26*	8.8±0.5	-9±4	14±5	24*
	Exendin-4	8.2±0.2	-17±3	19±3	36*	8.4±0.2	-16±2	15±2	31*
	Oxyntomodulin	6±0.4	-12±3	26±12	38*	6±0.4	-23±3	29±18	52*
GRK3 K220R	GLP-1(7-36)NH_2	7.8±0.2	-20±4	54±7	74*	7.9±0.2	-20±5	42±7	62*
	Exendin-4	8.3±0.2	-30±3	57±3	87	8.2±0.1	-28±3	55±3	83
	Oxyntomodulin	6.6±0.2	-23±4	68±10	91	6.7±0.2	-25±4	48±10	73
GRK5 K215R	GLP-1(7-36)NH_2	7.2±0.3	-5±4	41±6	46*	7.5±0.6	-21±8	13±10	34*
	Exendin-4	8.2±0.3	-30±8	18±6	48*	8±0.3	2±5	48±15	46*
	Oxyntomodulin	6.5±0.3	12±4	62±10	50*	6.3±0.3	14±6	81±9	67*
GRK6 K215R	GLP-1(7-36)NH_2	6.8±0.4	-6±4	34±11	40*	7.4±0.3	-13±4	26±6	39*
	Exendin-4	8±0.6	-35±11	4±9	39*	8.2±0.5	2±7	31±5	29*
	Oxyntomodulin	6.3±0.2	12±2	45±7	57*	6.4±0.2	2±3	58±6	56*

recruitment to the GLP-1R and differential effects on the mechanism for oxyntomodulin-mediated β-arrestin recruitment compared to that mediated by GLP-1(7-36)NH_2 and exendin-4.

Small Molecule Ligands and Peptides Differentially couple the GLP-1R to Cellular Effectors

As mentioned in previous chapters, the GLP-1R is well known to couple to multiple intracellular signalling components and therefore has the ability to engender stimulus bias towards particular pathways. This bias can be easily visualized in bias plots that plot the equipotent responses of one pathway versus another (Figures 6.23-6.26). Bias plots were therefore generated for each of the three peptide ligands, Compound 2 and BETP. Plotting each of the following pathways; cAMP, ERK1/2, Ca^{2+}, β-arrestin1 and β-arrestin2 against recruitment of GRKs 2,3 and 5, demonstrated that with the exception of BETP, each ligand coupled much more strongly to cAMP production than GRK recruitment (Figure 6.23 A - 6.25 A). BETP displayed bias towards GRK recruitment. However, in these plots, compared to the peptide ligands Compound 2 appears less biased towards cAMP compared to GRK5 (Figure 6.25 A).

GRK2 and GRK5 displayed similar patterns of bias in these plots. Analysis of GRK2 and GRK5 recruitment against the other signalling pathways, pERK1/2 Ca^{2+} and β-arrestin1 and β-arrestin2 recruitment, revealed both BETP and Compound 2 are more biased towards GRK2 and GRK5 recruitment (Figure 6.23 and Figure 6.25 B-E). There is, however, more variability in the relative signal bias of the peptides across the other pathways. In comparison to the primary endogenous agonist GLP-1(7-36)NH_2, both exendin-4 and oxyntomodulin appear more biased towards pERK1/2 than GRK2 or GRK5, while exendin-4, but not oxyntomodulin also appears more biased towards β-arrestin1 and β-arrestin2 recruitment over GRK2 or GRK5 (Figures 6.23

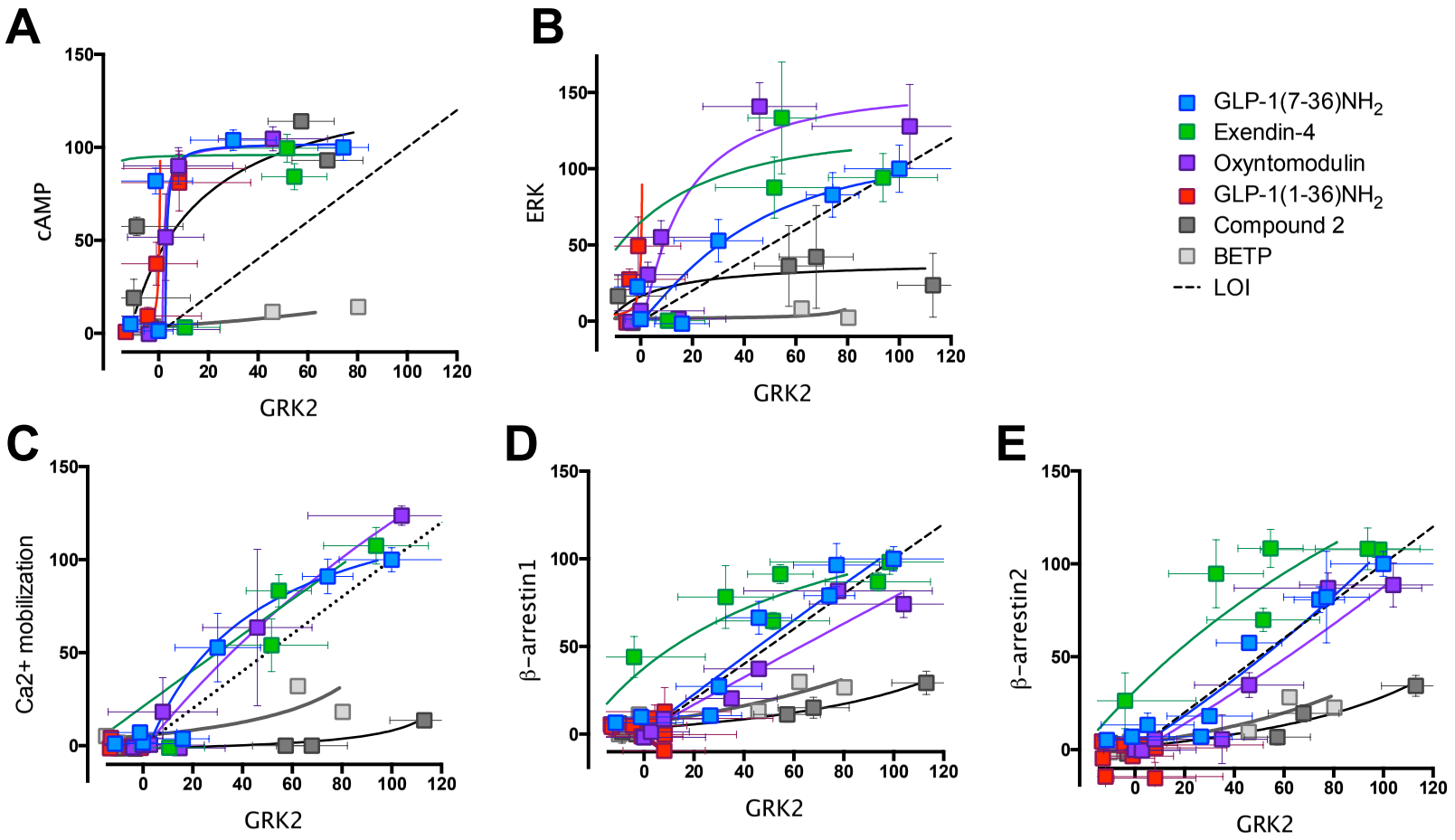

Figure 6.23 Both GLP-1R small molecules and peptides display stimulus bias relative to endogenous ligand GLP-1(7-36)NH_2.

Bias plots of GRK2 versus cAMP (A), GRK2 versus pERK1/2 (B), GRK2 versus Ca^{2+} mobilization (C), GRK2 versus β-Arrestin1 (D), GRK2 versus β-Arrestin2 (E). Concentration response data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve. Equipotent concentrations of ligand and the curve derived from the analysis with the three parameter equation were then plotted against each other for each pathway combination.

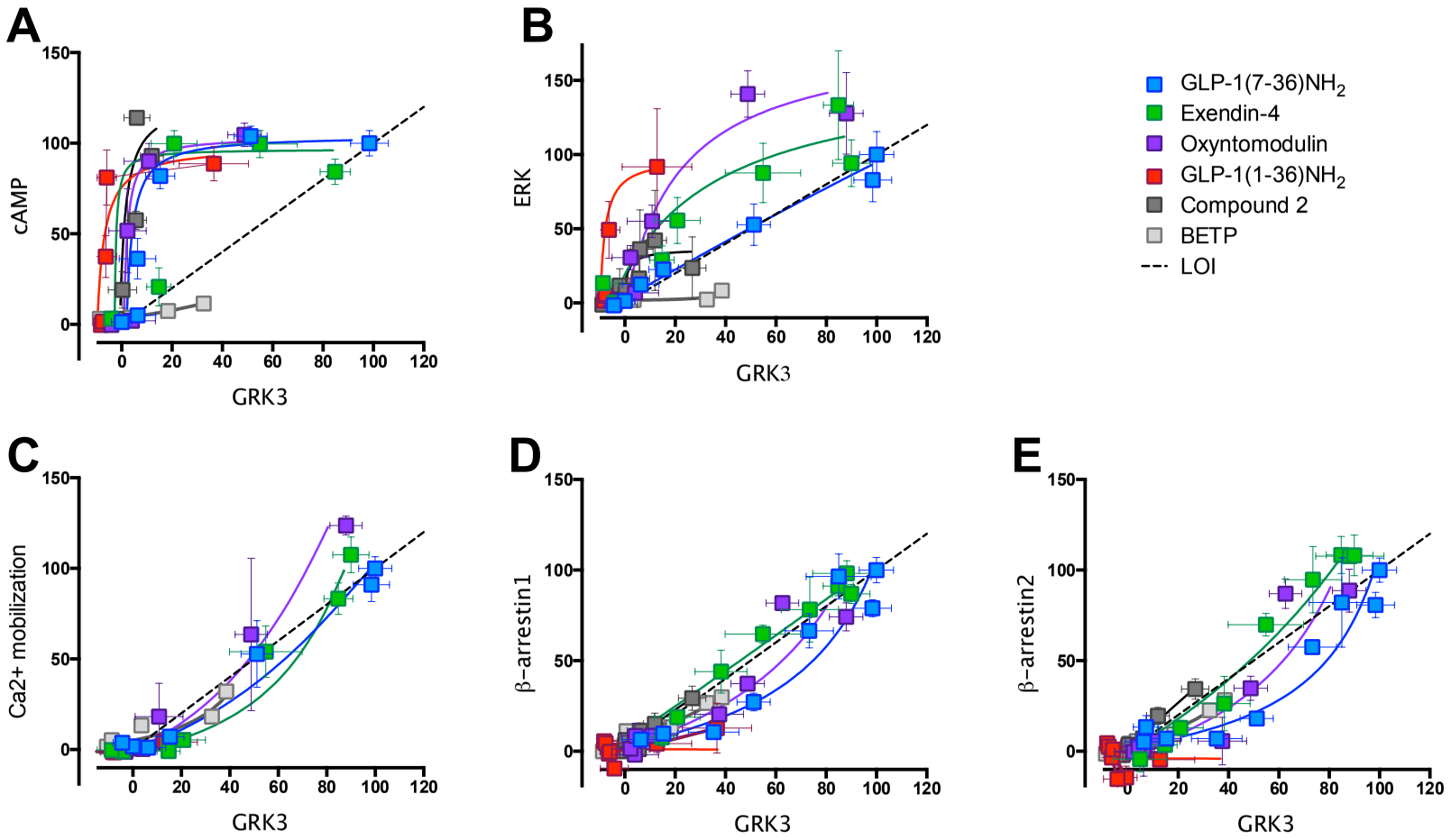

Figure 6.24 Both GLP-1R small molecules and peptides display stimulus bias relative to endogenous ligand GLP-1(7-36)NH2.

Bias plots of GRK3 versus cAMP (A), GRK3 versus pERK1/2 (B), GRK3 versus Ca^{2+} mobilization (C), GRK3 versus β-Arrestin1 (D), GRK3 versus β-Arrestin2 (E). Concentration response data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve. Equipotent concentrations of ligand and the curve derived from the analysis with the three-parameter equation were then plotted against each other for each pathway combination.

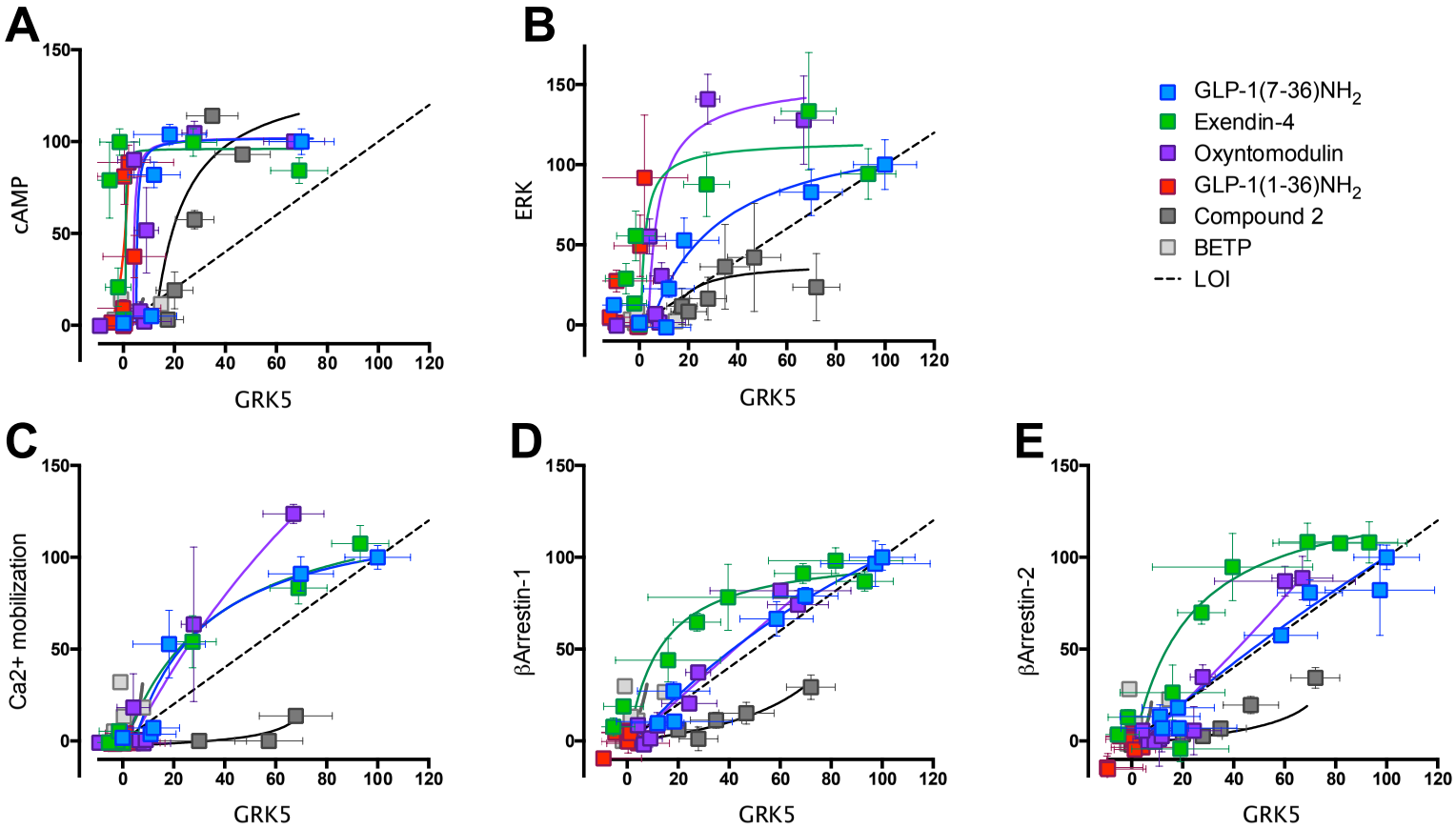

Figure 6.25 Both GLP-1R small molecules and peptides display stimulus bias relative to endogenous ligand GLP-1(7-36)NH_2.

Bias plots of GRK5 versus cAMP (A), GRK5 versus pERK1/2 (B), GRK5 versus Ca^{2+} mobilization (C), GRK5 versus β-Arrestin1 (D), GRK5 versus β-Arrestin2 (E). Concentration response data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve. Equipotent concentrations of ligand and the curve derived from the analysis with the three-parameter equation were then plotted against each other for each pathway combination.

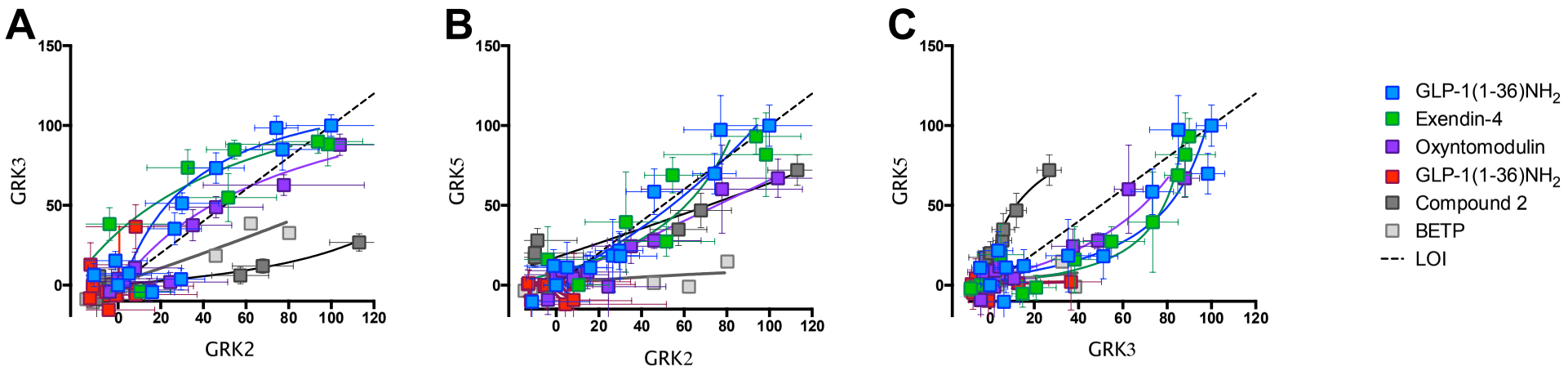

Figure 6.26 Both GLP-1R small molecules and peptides display stimulus bias relative to endogenous ligand GLP-1(7-36)NH_2.

Bias plots of GRK2 versus GRK3 (A), GRK2 versus GRK5 (B), GRK3 versus GRK5 (C). Concentration response data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve. Equipotent concentrations of ligand and the curve derived from the analysis with the three parameter equation were then plotted against each other for each pathway combination.

and 6.25 B, D and E). However, there appears no relative bias between the different peptides for Ca^{2+} mobilization compared to GRK2 or GRK5 (Figure 6.23 C).

A different pattern of bias is observed for GRK3 when plotted against the other pathways. In this system, GLP-1(7-36)NH_2 has no bias between pERK1/2 or Ca^{2+} and GRK3 as these lie on the LOI. The other ligands display a similar pattern for Ca^{2+}, however, in comparison to GLP-1(7-36)NH_2, the other ligands display some relative bias for pERK1/2 with Compound 2, exendin-4 and oxyntomodulin demonstrating bias towards pERK1/2 and BETP bias towards GRK3 (Figure 6.24 B, C). There are little differences between all ligand curves when β-arrestin1 and β-arrestin2 recruitment were plotted against GRK3, however there may be some relative bias for all ligands towards β-arrestin recruitment compared to GLP-1(7-36)NH_2.

GLP-1(1-36)NH_2 did not engender any detectable recruitment of GRKs (with the exception of GRK3), however it does signal via cAMP and pERK1/2. Due to the lack of detectable GRK2, 5 and 6 recruitment via this peptide, this implies that GLP-1(1-36)NH_2 is biased towards cAMP and pERK1/2. For GRK3 where recruitment was detectable, there appears to be heavy bias towards pERK1/2 (away from GRK3 recruitment) compared to GLP-1(7-36)NH_2 with no relative bias between GRK3 and cAMP compared to the other peptides (Figure 6.24)

Interestingly, assessment of the various GRKs plotted against one another revealed peptide ligands are more strongly coupled to GRK3 than GRK2 and 5 in this system, however relative to this, BETP and Compound 2 and more strongly coupled to GRK2 than GRK3, whereas Compound 2, but not BETP is more strongly coupled to GRK5 than GRK3 (Figure 6.26 A and C). When comparing GRK2 to GRK5, there is little preference for peptides and Compound 2 between these GRKs, but BETP is more strongly coupled to GRK2 (Figure 6.26 B).

Despite these bias plots being very convenient to visually observe the relative bias of different ligands, it not possible to apply quantitative analysis bias through this method. Instead, this can be assessed by the calculation of bias factors. Calculation of these bias factors revealed that due to the propagation of errors, very large changes in bias are needed to be observed for this bias to reach significance (Figures 6.27-6.30, Table 6.5). Although there were many trends towards bias, calculated bias factors showed significance ($p < 0.05$) only for BETP-mediated recruitment towards GRK2 and GRK5 in comparison to pERK1/2 signalling pathway (Figure 6.27 B and 6.29 B; Table 6.5). The generation of larger population numbers within these experiments may reduce the propagation of error such that bias identified in the bias plots may become quantitatively significant.

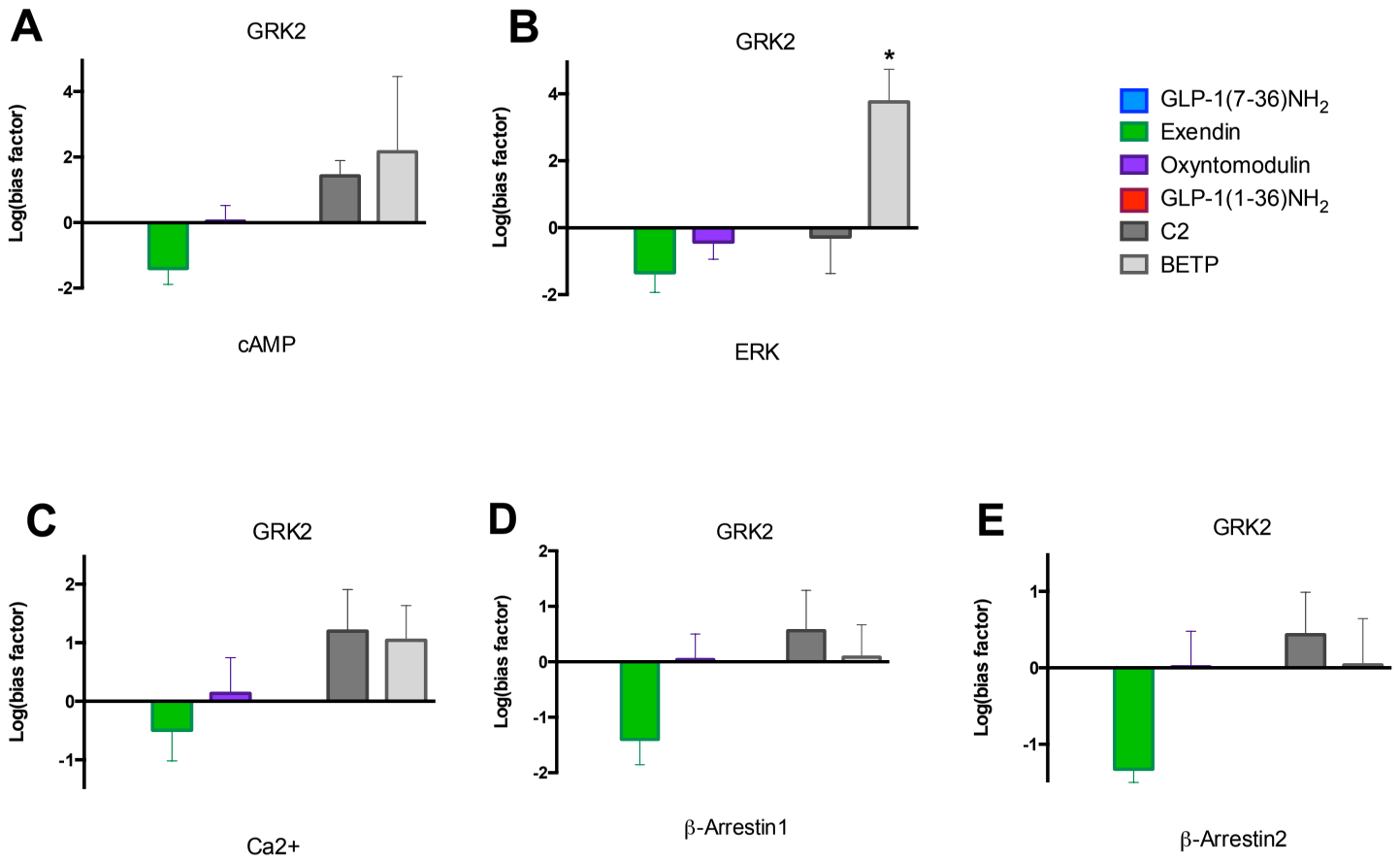

Figure 6.27 Biased signalling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.

Data were analysed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). GRK2 versus cAMP (A), GRK2 versus pERK1/2 (B), GRK2 versus Ca^{2+} mobilization (C),GRK2 versus β-Arrestin1 (E), GRK2 versus β-Arrestin2. Data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2. Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

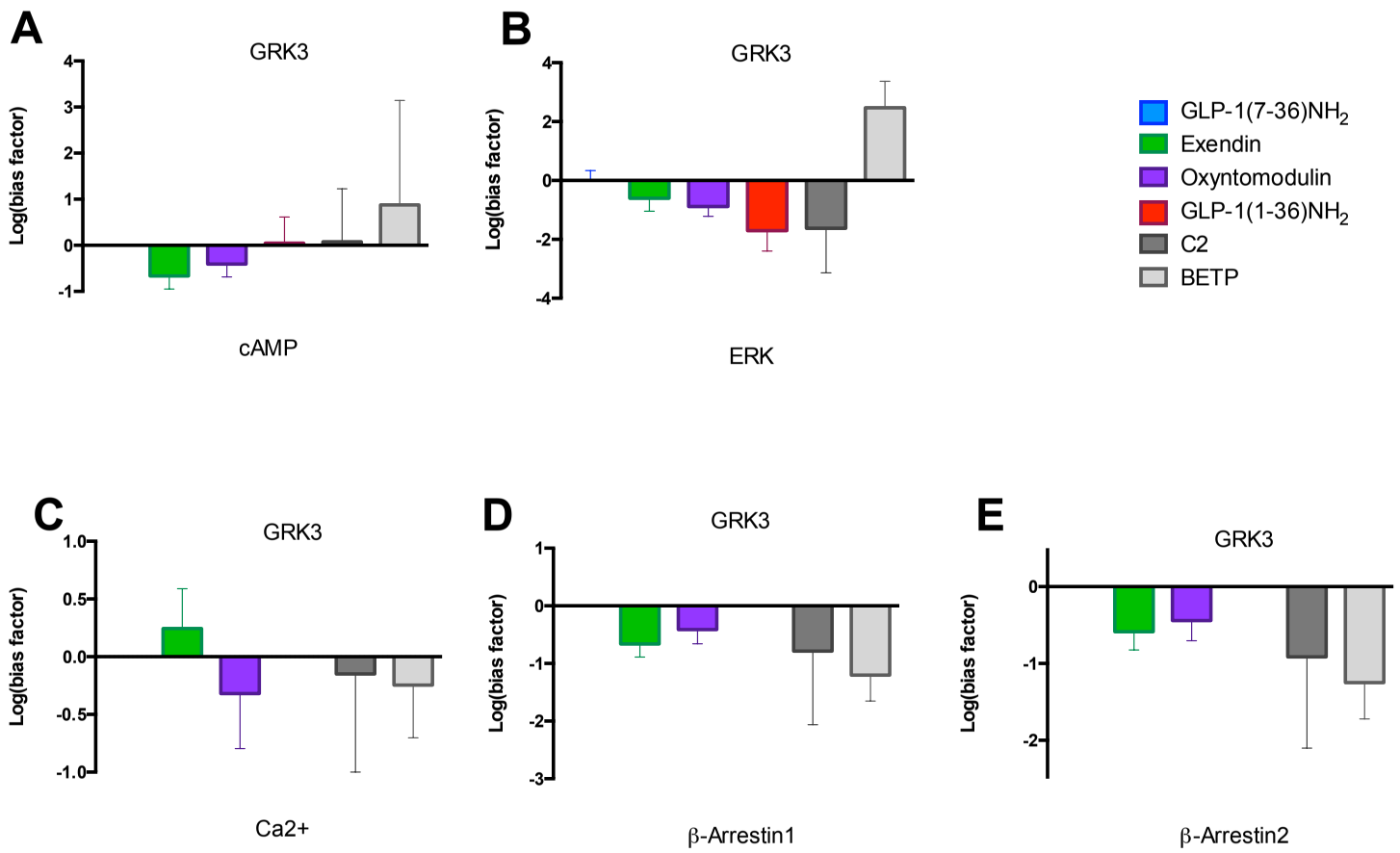

Figure 6.28. Biased signalling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.

Data were analysed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). GRK3 versus cAMP (A), GRK3 versus pERK1/2 (B), GRK3 versus Ca^{2+} mobilization (C), GRK3 versus β-Arrestin1 (E), GRK3 versus β-Arrestin2. Data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2. Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

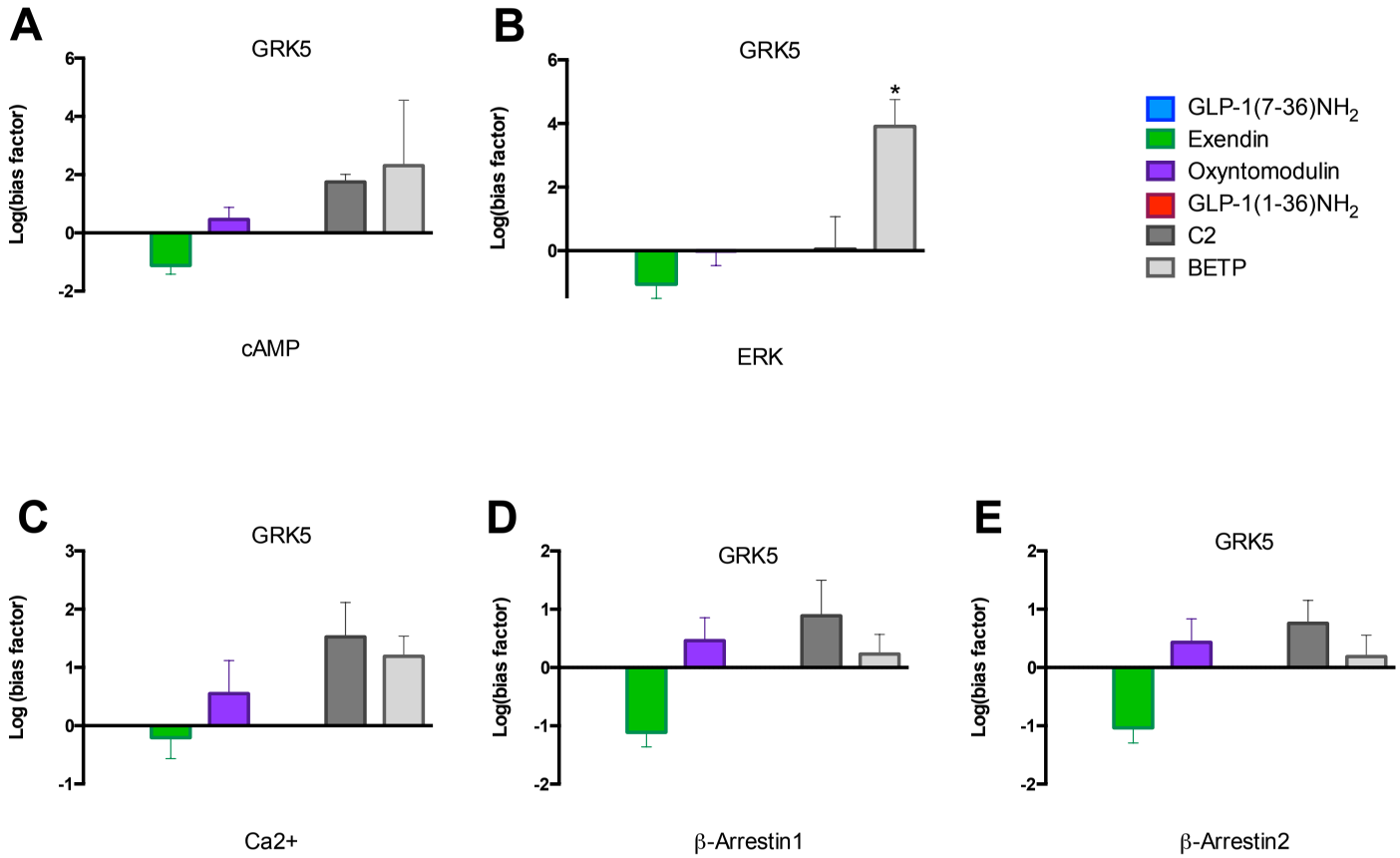

Figure 6.29 Biased signalling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.

Data were analysed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). GRK5 versus cAMP (A), GRK5 versus pERK1/2 (B), GRK5 versus Ca^{2+} mobilization (C),GRK5 versus β-Arrestin1 (E), GRK5 versus β-Arrestin2. Data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2. Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

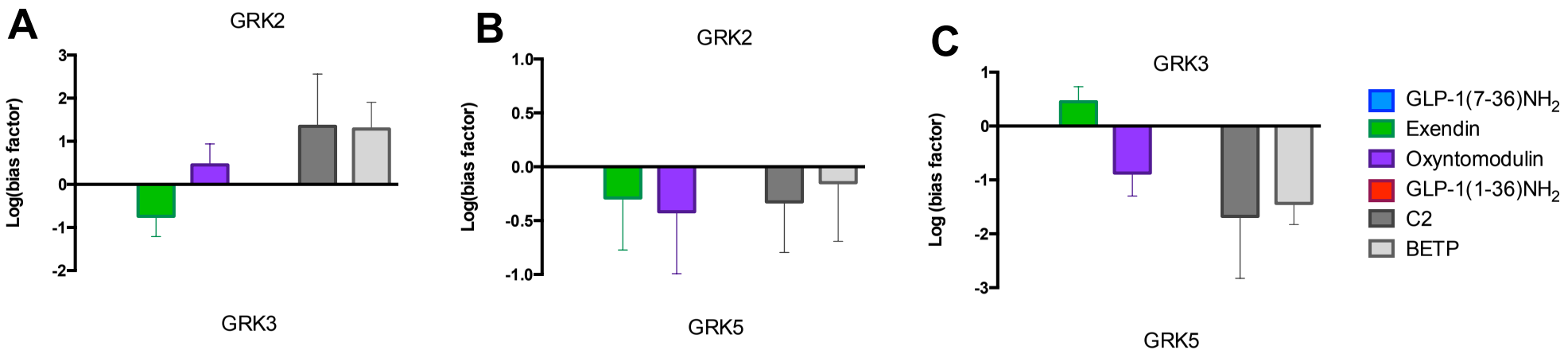

Figure 6.30 Biased signalling exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.

Data were analysed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). GRK2 versus GRK3 (A), GRK2 versus GRK3 (B), GRK3 versus GRK5 (C). Data for each ligand pathway were normalized to the maximal response elicited by GLP-1(7-36)NH_2. Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

Table 6.5 Stimulus bias exhibited by ligands relative to the reference agonist GLP-1(7-36)NH_2.
Data were analyzed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios, τ_c/K_A ratios are expressed in brackets Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signalling pathways relative to that of the reference agonist (GLP-1(7-36)NH_2). Values are expressed as means ± S.E.M. of three to 5 independent experiments conducted in duplicate. *Data are statistically significant at $p < 0.05$, one-way analysis of variance, and Dunnett's post test in comparison with reference agonist GLP-1(7-36)NH_2.

Pathway : Pathway	GLP-1(7-36)NH_2	Exendin	Oxyntomodulin	GLP-1(1-36)NH_2	Compound 2	BETP
GRK2: cAMP	0±0.49(1)	-1.41±0.48(25.41)	0.05±0.48(0.9)	-	1.43±0.47(0.04)	2.16±2.3(0.01)
GRK2: pERK1/2	0±0.55(1)	-1.35±0.59(22.13)	-0.43±0.51(2.69)	-	-0.28±1.09(1.88)	3.76±0.97(0.00)*
GRK2: Ca^{2+}	0±0.5(1)	-0.5±0.52(3.13)	0.13±0.61(0.73)	-	1.2±0.71(0.06)	1.04±0.59(0.09)
GRK2:β-arrestin1	0±0.49(1)	-1.4±0.45(25.06)	0.04±0.45(0.27)	-	0.56±0.72(0.27)	0.09±0.58(0.82)
GRK2:β-arrestin2	0±0.49(1)	-1.33±0.46(21.13)	0.01±0.47(0.97)	-	0.43±0.55(0.37)	0.04±0.6(0.91)
GRK3:cAMP	0±0.23(1)	-0.67±0.28(4.63)	-0.41±0.27(2.56)	0.05±0.57(0.89)	0.08±1.15(0.84)	0.88±2.27(0.13)
GRK3:pERK1/2	0±0.34(1)	-0.61±0.43(4.03)	-0.88±0.34(7.66)	-1.71±0.79(50.7)	-1.62±1.52(41.98)	2.47±0.9(0.00)
GRK3:Ca^{2+}	0±0.36(1)	0.24±0.35(0.57)	-0.32±0.48(2.09)	-	-0.15±1.27(1.41)	-0.25±0.46(1.76)
GRK3:β-arrestin1	0±0.22(1)	-0.66±0.23(4.57)	-0.41±0.24(2.58)	-	-0.79±1.27(6.11)	-1.2±0.45(15.9)
GRK3:β-arrestin2	0±0.23(1)	-0.59±0.24(3.85)	-0.44±0.26(2.76)	-	-0.91±1.19(8.20)	-1.25±0.47(17.7)
GRK5:cAMP	0±0.26(1)	-1.11±0.3(13.06)	0.46±0.42(0.34)	-	1.75±0.26(0.02)	2.3±2.25(0.00)
GRK5:pERK1/2	0±0.35(1)	-1.06±0.45(11.37)	-0.01±0.46(1.03)	-	0.05±1.02(0.39)	3.91±0.85(0.00)*
GRK5:Ca^{2+}	0±0.37(1)	-0.21±0.36(1.61)	0.55±0.56(0.28)	-	1.52±0.59(0.03)	1.19±0.34(0.06)
GRK5:β-arrestin1	0±0.25(1)	-1.11±0.25(12.88)	0.46±0.39(0.35)	-	0.89±0.61(0.13)	0.23±0.33(0.58)
GRK5:β-arrestin2	0±0.25(1)	-1.04±0.26(10.86)	0.43±0.4(0.37)	-	0.76±0.39(0.17)	0.19±0.37(0.17)
GRK2:GRK3	0±0.51(1)	-0.74±0.47(5.48)	0.45±0.48(0.35)	-	1.35±1.21(0.45)	1.29±0.62(0.05)
GRK2:GRK5	0±0.52(1)	-0.29±0.48(1.95)	-0.42±0.57(2.62)	-	-0.33±0.47(2.12)	-0.15±0.54(1.41)
GRK3:GRK5	0±0.28(1)	0.45±0.28(0.35)	-0.87±0.43(7.45)	-	-1.67±1.15(47.21)	-1.44±0.39(27.23)

Discussion

GPCR regulation is complex and involves processes including desensitisation, internalisation, recycling and downregulation of receptors. The classical paradigm for these processes involves GPCR phosphorylation by GRKs followed by β-arrestin binding, uncoupling of G protein signalling and internalisation of arrestin bound receptors complexes (Lefkowitz 2013a). In reality, this process is a lot more complex for most GPCRs(Kohout & Lefkowitz 2003). Extensive research in recent years has focused on the interactions between GPCRs and regulatory proteins, such as β-arrestins and GRKs, and the consequences of these interactions with regards to receptor signalling, regulation and the resultant physiological outcomes.

There are multiple GRKs, four of which (GRK 2,3,5 and 6) are ubiquitously expressed throughout the body (Ribas et al. 2007). Emerging studies have revealed significant influences of these GRK proteins on cellular function and they may have roles in disease onset, progression or in therapeutic targeting for some disorders. For example, inhibition of GRK2 at the βAR has shown promising effects as a treatment against progression of heart failure (Cannavo et al. 2013). GRK2 knockout studies revealed enhanced basal and adrenergic responses in cardiac function in adult heterozygotes (Rockman et al. 1998) and altered progression of experimental autoimmune encephalomyelitis (Vroon et al. 2005). Studies that investigated the effects of GRK3 knockdown have found a deficiency of olfactory receptor desensitization (Peppel et al. 1997), altered M_2 muscarinic airway regulation (Walker, Peppel, et al. 1999a), blunted kappa-opioid receptor–mediated tolerance in spinal analgesia test (M. Xu et al. 2004; McLaughlin et al. 2004), and disturbed tolerance to the antinociceptive effects of fentanyl but not morphine (Terman et al. 2004), reviewed by (Premont & Gainetdinov 2007). Furthermore, GRK3 influences opioid analgesic tolerance but not opioid withdrawal(Terman et al. 2004). GRK5 knockout studies have shown altered central (Gainetdinov et al. 1999) and lung (Walker et al.

2004) M_2 muscarinic acetylcholine receptor (M_2R) regulation, with normal heart M_2R regulation (Walker et al. 2004). In addition, there was pronounced behavioural and biochemical muscarinic super-sensitivity in GRK5-deficient mice (Gainetdinov et al. 1999). GRK6 deficient mice displayed altered central DAR regulation with interruption of DAR desensitization and behavioural abnormalities (Gainetdinov et al. 2003) (Seeman et al. 2005), deficient lymphocyte chemotaxis (Fong et al. 2002), increased acute inflammation and neutrophil chemotaxis (Kavelaars et al. 2003; Vroon et al. 2004) reviewed by (Premont & Gainetdinov 2007). These studies identify that GRKs are important in GPCR-mediated cellular functions and that altering their ability to function can result in onset or progression of disease.

There is evidence to show GRK specificity for particular GPCRs, such that a GPCR may not be regulated similarly by all GRKs expressed in a cell, with evidence that some GPCRs are functionally paired with a particular GRK (Fong et al. 2002). For example, activation of β2AR by isoproterenol results in phosphorylation by both GRK2 and GRK6, while stimulation by carvedilol (weak β-arrestin-biased agonists) only causes phosphorylation by GRK6 (Nobles et al. 2011). Despite some evidence for GRK interaction with the GLP-1R, the physiological functions of interaction with individual GRKs has not been thoroughly explored. The above studies emphasise the importance of investigating the roles of multiple GRKs in GPCR-mediated cellular functions and disease onset. As the evidence currently available in the literature supports that the GLP-1R can interact with more than one GRK (Jorgensen et al. 2011; Jorgensen et al. 2007; Jorgensen et al. 2005), it is important to fully profile the repertoire of GLP-1R interactions with these regulatory proteins and to elucidate their roles in cell signalling.

There are limited studies that have previously investigated the roles of β-arrestins and GRK interactions at the GLP-1R. To date, oxyntomodulin and glucagon have been observed to be agonists in recruiting both arrestins and GRK2 to the GLP-1R (Jorgensen et al. 2007). In

addition, there is evidence that β-arrestin2 competes with GRK2 for interaction with the activated GRK phosphorylated GLP-1R, suggesting a new role of β-arrestin2 in regulating the orchestration of GRK2 functionality (Jorgensen et al. 2011). Furthermore, one study revealed that GRK5 potentiates recruitment of β-arrestin2 to the receptor in response to GLP-1(7-36)NH_2 . This study also found that overexpression of GRK5 alone or β-arrestin2 and GRK5, significantly increased the GLP-1-induced internalization (Jorgensen et al. 2005). Although recruitment of GRK2 and -5 have been reported for this receptor, the recruitment profile of regulatory proteins to the GLP-1R in response to multiple endogenous, exogenous and small molecules ligands has not yet been fully explored. In addition, there are no studies assessing the ability of the receptor to interact with GRK3 and GRK6, and no studies assessing the effect of allosteric ligands.

In this study, we identified that GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin can transiently recruit GRK2, -3 and -5 to the GLP-1R while GLP-1(1-36)NH_2 solely promotes GRK3 recruitment (Figure 6.9). In addition, both small allosteric molecules Compound 2 and BETP were able to stimulate recruitment of all four GRKs (GRK2, -3, -5 and -6) (Figure 6.10). The ability of GLP-1(1-36)NH_2 to recruit GRK3 and not other GRKs could be due to experimental limitations and/or receptor occupancy at the maximum concentration used. For example, if the ligand had weaker efficacy for recruitment of these pathways, then a higher concentration (greater than 1μM that gives only approximately 50 % receptor occupancy (Koole et al. 2011)), may be required to enable detection of GRK interactions. However, the ability to detect recruitment by the small molecules Compound 2 and BETP at 3μM suggests that if GLP-1(1-36)NH_2 could recruit at concentrations higher than those tested in this study, it would be less efficacious than all these ligands, as at this concentration of small molecule, the receptor occupancy would be similar to GLP-1(1-36)NH_2 at 1μM (Wootten et al. 2013).

Despite the ability of the tested ligands to recruit the same GRKs, there is clear evidence for ligand-directed stimulus bias when considering the recruitment of distinct GRKs and this may have physiological significance. This is exemplified by studies on other GPCRs that suggest distinct downstream consequences for different GRKs acting at the same receptor. For numerous receptors, coupling to GRK2 and GRK3 results in phosphorylation and arrestin recruitment leading to desensitization, whereas GRK5 and 6 lead to β-arrestin mediated signalling (J. Kim et al. 2005; Ren et al. 2005). This has been shown for the $AT_{1A}R$, whereby by GRK2 and GRK3 induce receptor desensitisation and internalisation, while GRK5 leads to β-arrestin-dependent pERK1/2 activation (J. Kim et al. 2005). This has also been exemplified at the V_2R, where GRK2 and GRK3 promote desensitisation, while GRK5 and GRK6 are responsible for pERK1/2 downstream signalling (Ren et al. 2005). However, this is not always the case as there are also emerging studies that indicate GRK5 and GRK6 can be primarily involved in desensitization for some GPCRs. For example, calcitonin gene-related peptide receptors (Aiyar et al. 2000) and dopamine D_{1A} receptors(Tiberi et al. 1996). In addition transgenic mice overexpressing GRK5 in a cardiac specific manner show a marked enhancement of β-AR desensitization (Rockman et al. 1996). Reports have also revealed that different CCR7-CC chemokine ligands CCL19 and CCL21 activated distinct GRKs that resulted in different signalling outcomes. CC19 induced receptor desensitisation through activation of GRK3 and GRK6, whereas CCL21 promoted GRK/β-arrestin-mediated signalling that was dependent on GRK6 alone (Zidar et al. 2009). Other studies found biased agonists of the $AT_{1A}R$, TRV120023 and TRV120027, that increased cardiac contractility *in vitro* and *in vivo* (Violin et al. 2010; K. Rajagopal et al. 2006), but interestingly, only TRV120023 promoted the survival of cardiomyocytes during ischemia/reperfusion injury *in vivo* (K.-S. Kim et al. 2012).The biased agonist TRV120023 promoted GRK/β-arrestin-dependent signalling by $AT_{1A}R$, however it remains to be determined which GRKs are involved in $AT_{1A}R$-mediated biased signalling(Watari

et al. 2014).

These studies highlight the delicate interplay between different GRKs and how they may regulate the balance of G protein and β-arrestin-mediated signalling pathways downstream of GPCRs (Heitzler et al. 2012). These studies introduce the "barcode" hypothesis whereby specific and distinct patterns of receptor phosphorylation by individual GRKs are coupled with distinct functions of β-arrestins. Therefore the proposal is that the distinct phosphorylation patterns established by different GRKs acting at the same receptor establish a "barcode" that imparts distinct conformations to the recruited β-arrestin, thus regulating its functional activities (Nobles et al. 2011; Tobin 2009).

For GLP-1R, GRK2 and GRK5 are important for recruitment of both β-arrestin1 and β-arrestin2 as the overexpression of dominant negative versions GRK2 and GRK5 impaired ligand-mediated recruitment of these arrestins to the receptor. Important questions remain as to whether these GRKs phosphorylate the GLP-1R in an equivalently way, allowing for the same downstream functions upon β-arrestin binding or whether the two GRKs are involved in inducing differential phosphorylation patterns that lead to differential downstream consequences following the binding of arrestins. For example, does the involvement of one GRK lead to β-arrestin mediated signalling and the other to β-arrestin-mediated desensitization as has been observed for other GPCRs? Studies at the V2R have proposed GRK2 and/or GRK-3-phosphorylation drives high affinity binding of β-arrestins, while GRK5 and/or GRK-6 phosphorylated V2R may adopt an alternative conformation that induces β-arrestin2-mediated pERK1/2 signalling (Ren et al. 2005). There is limited evidence suggesting a role for GRK5 in GLP-1R internalisation, however there are also reports that GLP-1R internalisation occurs independent of β-arrestin recruitment

(Syme et al. 2006). Further work needs to be performed to address these important questions.

GRK3 recruitment to the GLP-1R (and presumably phosphorylation) does not appear to be involved in the recruitment of β-arrestins as overexpression of a dominant negative form of GRK3 had no effect on ligand-mediated GLP-1R β-arrestin recruitment (Figure 6.19-20) as previously supported by (Horie & Insel 2000). However, GRK3 was strongly recruited to the receptor by all ligands investigated (Figure 6.9-10), suggesting that it is important in receptor function. Whether GRK3 plays a role directly in signalling or in regulation of the receptor signal still needs to be determined. It could be speculated that GRK3 could play a potential role in GLP-1R desensitisation/internalization that is independent of β-arrestins; as noted above as there are studies that suggest GLP-1R-internalisation is a β-arrestin independent process (Syme et al. 2006). There is evidence for this in other receptor systems, where GRK3 plays a pivotal role in CXCR4 signalling in leukocytes of WHIM syndrome patients, with evidence to support that GRK3 binding directly to G proteins and potentially contributing to CXCR4 desensitization in a phosphorylation and/or β-arrestin-independent manner (Balabanian et al. 2008). This study on the CXCR4 receptor provides evidence of internalization that is dependent on GRK3, but independent of β-arrestin recruitment, thus providing alternative avenues that could be explored for the role of GRK3 in GLP-1R signalling/regulation. Further work assessing the role of GRKs 2, 3 and 5, as well as β-arrestins in GLP-1R signalling, internalisation and desensitization will need to be explored to fully understand the role of these different GRKs in GLP-1R function.

Studies assessing β-arrestin recruitment also revealed a role of GRK6, as overexpression of dominant negative GRK6 reduced both β-arrestin1 and β-arrestin2 recruitment to the GLP-1R. However in our GRK6 recruitment assay, we were unable to detect any GRK6 recruitment. This discrepancy could be due to experimental limitations where we may have been unable to detect the low level of membrane bound GRK6 recruitment due to the high net BRET in the system, or

perhaps due to the positioning of the tags in the receptor and GRK6 not being in optimal positions to detect a BRET response. However, this is unlikely given that this was not the case for the GRK5 construct (the other membrane bound GRK). An alternative explanation for the inability to detect GRK6 recruitment is that activation of other kinases such as PKA or PKC, that are reported to be activated by the by GLP-1R are potentially involved in GRK6 regulation. These second messenger kinases have been shown to regulate the internalisation of family B receptors (Bisello et al. 2004; Ferrari et al. 1999; Walker, Premont, et al. 1999b). Recent reports are revealing that a number of proteins interact with GRKs, thus unveiling novel mechanisms of regulation and therefore altering downstream consequences of signalling of GPCRs (Ribas et al. 2007). While there is evidence for expression of GRK6 in human pancreatic islets (Eizirik et al. 2012), GRK6 is ubiquitously expressed throughout the body in other regions where the GLP-1R is present and where interactions with GRK6 might be evident (Eizirik et al. 2012).

This current chapter revealed that Compound 2 and BETP display probe dependence in their abilities to modulate ligand-induced GRK function. We showed differential abilities of the allosteric ligands to modulate recruitment of GRKs depending on the orthosteric ligand being assessed, but also that these ligands promoted different levels of modulation on the recruitment of distinct GRKs when activated by the same ligand. For GRK2 recruitment, all peptides were positively modulated by both BETP and Compound 2, albeit to different extents, with oxyntomodulin not reaching statistical significance (Table 6.5). However, for GRK3, only oxyntomodulin and GLP-1(7-36)NH_2 showed significant levels of positive modulation and this was only observed with Compound 2, but not BETP. For GRK5 recruitment, there was no modulation of any peptide by either BETP or Compound 2. It is therefore extremely important to begin to elucidate the physiological roles of these distinct GLP-1R-GRK interactions in terms of GLP-1R signalling and regulation, especially for future development of allosteric ligands in drug

discovery programs, as these types of ligands introduce even more complexity into an already complex system.

Not only is it important to understand the modulation profiles of different ligands, but to understand the mechanism for the effects that are observed. For example, Compound 2 potentiates GRK3 recruitment by GLP-1(7-36)NH_2 and oxyntomodulin by 13-fold and 28-fold respectively. However, oxyntomodulin affinity is potentiated around 13-fold by Compound 2, whereas GLP-1(7-36)NH_2 affinity is not significantly altered (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a), suggesting that both ligands in this case undergo a similar extent of efficacy modulation for GRK3 recruitment by Compound 2. In the case of GRK2, both GLP-1(7-36)NH_2 and oxyntomodulin have similar levels of modulation implying that due to the positive binding cooperativity of oxyntomodulin, Compound 2 efficacy modulation is neutral with this peptide. However, in the case of GRK5, these ligands appear to have the same effect on function (as neither modulates), but given that oxyntomodulin affinity is enhanced by around 10-fold, this may imply that there is negative efficacy modulation for GRK5 by this ligand, but not by GLP-1(7-36)NH_2 or that functional affinity is different. These data imply that enhancing the affinity of oxyntomodulin enhances the efficacy of the receptor towards GRK3 at the expense of decreasing the efficacy of the receptor towards other GRKs compared to GLP-1(7-36)NH_2, implying that the co-occupation of the receptor with distinct orthosteric-allosteric ligand combinations induces distinct repertoires of receptor conformations that differentially influence interaction with downstream effectors. Within this chapter, there are multiple examples of this, such as differential extents of modulation of exendin-4 and GLP-1(7-36)NH_2 efficacy for GRK2 and GRK3 recruitment by Compound 2 and BETP, and the reversal in the rank order of positive modulation observed by Compound 2 with GLP-1(7-36)NH_2 and exendin-4 when comparing GRK2 and GRK3 recruitment. The differential effects of allosteric ligands to alter the ability of the receptor to

interact with specific GRKs may have some physiological relevance. For example, GRK2 and GRK5 play key roles in the $AT_{1A}R$ in desensitization of G-protein activation and β-arrestin-dependent signalling respectively. Recent studies showed that overexpression of GRK2 exerts a strong negative effect on β-arrestin-dependent signalling through its ability to compete with GRK5 and 6 for promoting receptor phosphorylation, (Heitzler et al. 2012), thus suggesting that differentially altering specific GRK function can dramatically alter the downstream consequences or the quality of signal from the activated GPCR. For the GLP-1R, this therefore requires further investigation to understand the downstream consequences of the differential modulation observed in this study by different orthosteric/allosteric ligand combinations. The ability of multiple combinations of allosteric/orthosteric ligands to provide such differences in their ability to differentially recruit distinct GRKs provides tools to address a range of questions, such as whether selectively modulating particular GRKs at the exclusion of others may provide novel therapeutic benefits.

In our studies we revealed a high net BRET signal for both GRK5 and GRK6 compared to GRK2 and GRK3 (Figures. 6.1-6.8). This could be attributed to the fact that both GRK5 and 6 are both membrane associated due to palmitoylation whereas GRK2 and GRK3 are cytosolic However studies have reported that it is possible for two membrane proteins to not producing a BRET signal when co-expressed. As BRET is a proximity assay and the GLP-1R-Rluc8 may be in close proximity to the membrane associated GRK5 and GRK6, this may account for the high net BRET. Furthermore the high basal BRET signal could indicate that there is already a complex between GRK5 and the receptor. In which case, the transient increase could indicate a conformational change and/or further recruitment of GRK5 to the receptor, while the subsequent decrease could result from a phosphorylation-dependent decrease in affinity and/or competition with endogenous β-arrestin molecules. Interestingly, in this chapter, the current work identified

unique kinetic profiles in the GRK5 studies. Immediately following receptor activation by ligand, a peak BRET response was observed, suggesting recruitment of GRK5. Following this there was a drop in BRET response that was significantly lower than baseline for the majority of ligands (Figure 6.5-6.6). To a smaller extent this drop in baseline was also observed in some of the GRK6 timecourses. This could be reflective of GLP-1R internalization or redistribution of the receptor within the plasma membrane following activation, whereby GLP-1R moves away from the plasma membrane while GRK5 remains membrane associated. All the peptide ligands assessed share this similar profile, but a smaller drop in baseline was observed for the small molecules, BETP and Compound 2. This may imply that these compounds perhaps are unable to internalise the receptor or at least not to the same extent or with the same kinetics as that of peptides (Coopman et al. 2010). Interestingly, co-addition of BETP, but not Compound 2 with peptide ligands, was able to inhibit the peptide-mediated drop in baseline, thus implying that Compound 2 and BETP may be exerting differential effects on the ability of peptide ligands to internalize, with BETP retaining the receptor at the plasma membrane (at least during the timecourse of the assay (20 min). At this stage these effects are speculative, but this could be measured and confirmed using a non proximity method such florescence microscopy using tagged receptors and possibly also with the addition of tagged ligands.

In conclusion this chapter characterized the recruitment profile of regulatory GRK-2, -3, -5 and -6 to the GLP-1R in response to endogenous, exogenous and small molecule compounds. Each GRK displayed distinct recruitment profiles when tested across all ligands. Furthermore, this chapter also investigated the different effects of GRKs on both β-arrestin1 and β-arrestin2 recruitment to the GLP-1R in response to different ligands and identified GRK-2, -5, and -6 play a role in β-arrestin recruitment, while GRK-3, although strongly recruited in response to all ligands tested did not influence β-arrestin recruitment to the GLP-1R.

In addition to identifying clear bias between the ability of distinct ligands to promote distinct GRK recruitment to the GLP-1R, comparisons between GRK recruitment profiles and other signalling pathways, including cAMP, Ca^{2+} mobilization and phosphorylation of ERK1/2, reveal that there are also very distinct biases induced between GRK recruitment and these other signalling pathways. Work from this chapter, the previous chapter (Chapter 5) and from data previously published in the Sexton lab also reveals distinct abilities of allosteric ligands to selectively modulate arrestin and GRK proteins and other classical signalling pathways, such as cAMP, Ca^{2+} and pERK1/2 in a probe-dependent manner (Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Wootten et al. 2013; Willard, Wootten, et al. 2012b) (Figures. 6.11-6.18). These studies highlight the complexities of the GLP-1R system and that by integrating small allosteric molecules with GLP-1R orthosteric agonists (many of which are found endogenously) the combined signaling output can be dramatically altered, and this could have major physiological implications.

It is evident that there is a need to identify the functional consequences for interactions of specific regulatory proteins at the GLP-1R and to identify the physiological relevance of these interactions. Furthermore it is essential to understand these physiological roles for future drug discovery and whether enhancement of specific GRK function by small allosteric molecules modulates downstream events that would be therapeutically beneficial by activating distinct signalling pathways in void of others. Although beyond the scope of this thesis, further investigations to assess the functional consequences of these different regulatory proteins have begun and involve knockdown of specific GRKs in a more physiological relevant cell line (an insulinoma cell line that endogenously expresses the GLP-1R (INS-1 832/13)). Specific functional endpoints being investigated in these knockdown models include proliferation, insulin

secretion, agonist-mediated inhibition of apoptotic events as well as classical signalling assays and receptor internalisation. Identifying and targeting the role of specific GRKs in these specific physiological outcomes would further advance development of future GLP-1R-mediated therapeutics for T2D.

CHAPTER 7:

General Discussion

GPCRs are critical in the regulation of many cellular processes that modulate physiological responses and are tractable drug targets for the treatment and management of many diseases (Ghanemi 2013). However, the concepts of biased signalling and allosteric modulation that have emerged in the field over the past decade have revealed increased complexity in how these receptors function, presenting both challenges and advantages in drug design and development.

For many years, ligand binding to a GPCR was considered to linearly affect all linked downstream signalling pathways and components that the receptor was able to couple to. However, more recently, studies exploring several signalling pathways have revealed that GPCR activity is pluridimensional, in that a ligand can possess distinct efficacies associated with selected signalling pathways arising due to the stabilization of multiple distinct conformational states of the receptor (Kenakin 2006; Kenakin et al. 2012). This concept has been termed ligand signalling biased (Kenakin 2012). There has been increasing interest in exploiting this behavior therapeutically, to selectively activate or suppress specific pathways to potentiate physiologically beneficial signalling events but eliminate events of receptor activity that may be associated with deleterious side effects.

In addition to exploiting biased signalling, the identification of allosteric compounds at GPCRs has gathered increasing interest in the pharmaceutical industry. Allosteric ligands offer many advantages over orthosteric ligands, with the ability to fine tune receptor responses, their capacity to provide selectivity amongst homologous GPCRs and their ability to modulate specific cellular events through their cooperativity with orthosteric ligands (Langmead & Christopoulos 2014). For these reasons, the pursuit of allosteric drugs has become a major focus of many drug discovery programs, however there are numerous challenges in both the identification of allosteric compounds in *in vitro* systems and their translation and ability to

fulfill their desired effect *in vivo*. Screening programs for these ligands principally involve HTS using one signalling pathway and often the most physiologically relevant ligand, and although this has been successful in some cases, there is an extremely high attrition rate in the number of new drugs identified (R. Zhang & Xie 2012). This is thought to reflect a lack of mechanistic understanding of the physiological basis and consequences of ligand directed signal bias and allosterism at GPCR targets. Many of these problems arise as screening programs generally do not take into account the potential for biased signalling or the potential for probe-dependent effects, which is relevant for systems that have multiple endogenous ligands or where the probe used in screening is not the primary endogenous ligand. In addition, dissecting pathways that are required for beneficial signalling versus detrimental effects when targeting GPCRs in many diseases is problematic as the pathways defining these effects is not well understood. It is therefore necessary to develop a comprehensive understanding of receptor function, particularly with respect to biased signalling and allosteric modulation, to maximize the translational potential of drug discovery and development programs.

This thesis explores signalling profiles of the GLP-1R in response to multiple ligands, both endogenous and exogenous, orthosteric and allosteric. The GLP-1R is a family B GPCR that is of major interest to the pharmaceutical industry for the treatment of type 2 diabetes and obesity (Drucker 2005; Burcelin et al. 2013; J. Liu et al. 2012a; Dalle et al. 2013). However, due to its expression in tissues other than the pancreas, it is also attracting interest as a potential target for the treatment of neurological diseases, such as Alzheimer disease (Y. Li et al. 2010) and cardiovascular diseases(Burgmaier et al. 2013). Previous studies have extensively explored the phenomena of biased signalling and allosteric modulation by multiple GLP-1R ligands in terms of cAMP accumulation, Ca^{2+} mobilization and pERK1/2 signalling (Koole, Wootten, Simms,

Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Koole et al. 2013; Wootten et al. 2012; Wootten et al. 2011). These studies demonstrate that distinct orthosteric and allosteric ligands display ligand-directed signalling bias that small molecule allosteric compounds acting at the GLP-1R can influence the effect of the orthosteric agonist acting at these pathways. Some of this work, although initially shown in recombinant cells lines, has been further validated in endogenously expressing GLP-1R cell lines as well as in *in vivo* animal models and in primary cells *ex vivo* (Appendix 1) (Willard, Wootten, et al. 2012b; Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010b; Wootten et al. 2013). In this PhD thesis, the current literature has been expanded to cover the extent to which ligand-directed signalling bias and allostery relates to recruitment of regulatory proteins and the metabolite of GLP-1 (that was considered to be an inert breakdown products of the endogenous GLP-1 ligands). Specifically, this thesis highlights the importance of exploring non-canonical pathways such as recruitment of regulatory proteins, when developing ligands therapeutically, as even ligands that can behave very similarly in classical signalling pathways are not always equivalent when other areas of receptor function are considered. In addition, it also highlights the importance of considering metabolic breakdown products of endogenous ligands when exploring allosteric interactions as these may have activity when in the presence of an allosteric ligand. These concepts may also apply to metabolic products of the drugs themselves that may have a different profile to those of the parental ligand when acting at the receptor in the presence of orthosteric ligands(Wootten et al. 2012; N. Li et al. 2012a).

The metabolite of endogenous GLP-1 (GLP-1(9-36)NH_2) binds to the GLP-1R with low affinity and does not produce detectable levels of cAMP (Wootten et al. 2012; N. Li et al. 2012a). In Chapter 3 (and to an extent Chapter 5), the ability of this "inert" metabolite to signal via other pathways (pERK1/2, iCa^{2+} mobilization and recruitment of the regulatory proteins, β-arrestin 1

and 2) was addressed. This revealed that while the GLP-1R metabolite GLP-1(9-36)NH_2 displays very weak ability to activate most signalling pathways, it can clearly activate downstream signalling to the pERK1/2 pathway. Assessment in Ins1-832 cells that endogenously express the GLP-1R, confirmed that GLP-1(9-36)NH_2 could activate pERK1/2 but that it cannot promote insulin secretion. This implies that pERK1/2 signalling alone is not sufficient to promote insulin secretion, and this is consistent with the literature, as cAMP is known to be essential for insulin secretion. Nevertheless, pERK1/2 activation has been linked to β cell survival (Sonoda et al. 2008) therefore it would be interesting to explore if the metabolite has the ability to promote cell survival, in the absence of the generation of cAMP, in order to dissect the importance of various signalling pathways to downstream physiological effects.

A substantial finding from this thesis was the ability of allosteric ligands to modulate the activity of the metabolite GLP-1(9-36)NH_2. Chapter 3 shows that Compound 2 and BETP both have the ability to induce a GLP-1(9-36)NH_2-mediated cAMP signal. Due to steepness of the hill slopes of the curves varying substantially between the GLP-1(9-36)NH_2 curves in the presence of different concentrations of allosteric ligand BETP (there is no curve for the metabolite alone, and the hill slope increases in steepness with increasing concentrations of allosteric compound), an operational model could not be applied to accurately predict a cooperativity factor, however, the level of modulation was substantial (Wootten et al. 2012). A similar finding was also observed in an independent study with significant modulation of cAMP when Compound 2 and the metabolite were coadded to HEK293 cells expressing the GLP-1R (N. Li et al. 2012a). Furthermore, we and Li *et al* also showed that this signalling profile of increased cAMP accumulation observed in recombinant cells was also detected in endogenously expressing GLP-1R cell lines (INS1-832 and INS-1E respectively) (Wootten et al. 2012). A key finding in our study was the demonstration that one of these compounds, BETP, could also strongly potentiate

insulin secretion by the metabolite in both *ex vivo* and *in vivo* models illustrating the principle that manipulation of the activity of metabolites could provide a therapeutic opportunity and should be considered in drug screening programs. In addition, chapter 3 also highlights the effects of modulating inert metabolites of other GPCRs; the M_2 mAChR and the A_1 –AR, which further demonstrated a potential avenue for metabolites as therapeutic targets for a broader range of GPCRs that may be relevant in multiple disease states (Wootten et al. 2012). Indeed this concept could also apply for other drug targets, such as ligand-gated ion channels.

In the study presented in chapter 3, we revealed that in CHOFlpIn cells expressing the GLP-1R, modulation of GLP-1(9-36)NH_2 by allosteric ligands Compound 2 and BETP only enhanced the activity of cAMP, while having little or no effect on pERK1/2 or iCa^{2+} mobilisation. From these studies, it could therefore be speculated that enhancement of cAMP alone without modulation of pERK1/2 or iCa^{2+} mobilisation may be sufficient for enhanced insulin secretion, however, a later study revealed that this may not be the case, and the bias profiling may differ in different cell backgrounds (N. Li et al. 2012a; Coopman et al. 2010). In addition to cAMP modulation, Li *et al* revealed enhancement of GLP-1(9-36)NH_2-mediated pERK1/2 and Ca^{2+} mobilization in HEK293 cells and enhancement of pERK1/2 in INS1-E cells by Compound 2. Thus, implying that the enhancement of insulin secretion may be due to an interplay of modulation of multiple pathways (N. Li et al. 2012a). The discrepancies between this study and the study presented in chapter 3 may arise for a number of reasons. Firstly, cell background may play a role, such that enhancement of iCa^{2+} mobilization and pERK1/2 could not be detected in the CHOFlpIn cells, but could in HEK293 cells. Secondly, as both of these systems are recombinant cells, receptor expression levels within a cell could play a role. A similar method for detection was used in both studies for Ca^{2+} mobilization, yet Li *et a*l using HEK293 cells could detect weak modulation by compound 2 of the metabolite, whereas no Ca^{2+} was detectable in the CHO cells used in this

thesis. However, the same group have also published data previously revealing detection of Compound 2 mediated Ca^{2+} responses at high concentrations that also could not be detected in studies described in this thesis (Coopman et al. 2010). This could be due to differences in cell background, but equally weak responses may be detected in studies where receptor density is higher although this cannot be confirmed as receptor density was not reported in the HEK293 study. Discrepancies between the ability/inability to detect pERK1/2 modulation is unlikely to be due to receptor density as Li *et al* were also able to detect pERK1/2 modulation in endogenously GLP-1R expressing INS1-E cell where receptor densities are likely to be significantly lower than in recombinant cell systems. Many different signalling proteins can lead to activation of pERK1/2 and the discrepancies between the study presented in Chapter 3 and Li *et al* may arise due to the different mechanisms leading to pERK1/2 activation being cell type dependent. The studies in this thesis were also all performed using an Alphascreen detection method that uses a very mild lysis, whereas the pERK1/2 in Li et al was detected using western blotting with pERK1/2 antibodies. This method may be more sensitive than the Alphascreen, or may even possibly detect a different pool of pERK1/2. Signalling components within a cell are highly regulated by compartmentalization, and pERK1/2 signals can equally be compartmentalized, for example pERK1/2 can be measured cytosolically or from nuclear compartments (Irannejad, Tomshine, et al. 2014b; Irannejad, Kotowski, et al. 2014a) (Tohgo et al. 2002) (Shenoy & Lefkowitz 2011). Discrepancies in the two studies may therefore represent distinct pools of pERK1/2, whereby one is modulated and the other not. This would be very interesting to explore in the future as activation of signalling in distinct regions within a cell may lead to different physiological effects.

Regardless of these differences between studies, both our study and that of Li *et al*, revealed the ability of small molecule allosteric compounds to modulate signalling by metabolites of GLP-1

that were previously considered inert using allosteric ligands. The ability of this to translate into enhanced insulin secretion provides strong evidence of 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.

The net effect of any receptor-based therapeutic reflects an interplay between acute signalling and the longer-term regulatory pathways (Lefkowitz 1998). Therefore, it is crucial to understand not only the acute effects of ligands, but also the chronic effects of ligands at their corresponding GPCR targets. GLP-1R orthosteric ligands that have been approved for therapeutic development are long lasting, more stable analogues of GLP-1, and allosteric modulators have also be proposed as potential therapeutics to manage diseases such as type 2 diabetes (Willard & Sloop 2012; Wootten et al. 2013). However, our understanding of their effects on receptor regulatory processes such as internalization and subcellular trafficking is still limited.

Upon activation, GPCRs can be rapidly phosphorylated on their intracellular face by GRKs (Willets, Challiss & Nahorski 2003a). This phosphorylation increases the affinity of the receptor for the scaffolding proteins β-arrestins, which associate with the GPCR and promote internalization (S. J. Perry & Lefkowitz 2002; Violin & Lefkowitz 2007). β-arrestins can also interact with multiple intracellular signalling partners and scaffold the receptor to distinct G protein-independent signalling pathways (Lefkowitz 2013a). Dissociation of β-arrestin from the GPCR occurs either at the cell surface or within endosomes, and the dynamics of such uncoupling have a profound impact on the subsequent fate of the desensitized receptor (Lefkowitz 2013a). Therefore these regulatory proteins (both GRKs and β-arrestin) can not only affect the length and strength of signals (by induction of internalization pathways and

scaffolding/uncoupling of signals) that are produced by at activated receptor, but also the location from which a receptor can signal and the extent of signalling pathways that the receptor can activate (S. J. Perry & Lefkowitz 2002).

In order to investigate recruitment of distinct regulatory proteins (arrestins and GRKs) to the GLP-1R, we developed a medium throughput system to investigate multiple ligands and their ability to recruit these proteins. This method, described in Chapter 4, allows us to use this system to assess interactions between different proteins where a potential interaction may occur. Therefore, this system may have broad utility for looking at GPCR regulatory interactions, as we have shown by testing three different receptor systems (V_2R, M_1 AChR, and GLP-1R). However, this technique could also have additional utility for assessing protein-protein interactions in any system, not just for GPCRs (Savage et al. 2013). Of these systems, the GLP-1R system was explored extensively, and the results are described in chapters 5 and 6.

From currently available literature, it is clear that both GLP-1R mediated Ca^{2+} mobilization and cAMP accumulation are strongly linked to insulin secretion in islets, while β-arrestin1-mediated pERK1/2 signalling downstream of GLP-1R activation is associated with β-cell survival (Sonoda et al. 2008). Prior to the studies presented in this thesis, the laboratory have performed detailed analysis of agonism in canonical signalling pathways that included cAMP accumulation, Ca^{2+} mobilization and pERK1/2 when the GLP-1R was activated by multiple ligands(Koole, Wootten, Simms, Valant, Sridhar, Woodman, Miller, Summers, Christopoulos & Sexton 2010a; Willard, Wootten, et al. 2012b; Wootten et al. 2012). This covered both endogenous and exogenous ligands and orthosteric and allosteric acting compounds. In addition to this, the ability of allosteric small molecule ligands to influence orthosteric ligand function had also been explored in these signalling outputs (Koole, Wootten, Simms, Valant, Sridhar, Woodman,

Miller, Summers, Christopoulos & Sexton 2010a; Willard, Wootten, et al. 2012b; Wootten et al. 2012; Wootten et al. 2011). These studies identified significant bias in the ability of distinct ligands to activate signalling pathways, in addition to probe-dependent effects of allosteric compounds to alter the signalling of orthosteric ligands. In Chapters 5 and 6, these signalling bias profiles of distinct ligands have been extended to include the recruitment of regulatory proteins β-arrestins and GRKs. The ability of allosteric ligands to alter orthosteric ligands in these pathways was also explored. These studies have demonstrated a wide profile by which different ligands engender distinct recruitment of particular GRKs and β-arrestins.

GLP-1R peptide ligands, GLP-1(7-36)NH_2, exendin-4 and oxyntomodulin all mediated recruitment of both β-arrestins and GRK-2, -3, and -5, however GLP-1(1-36)NH_2 only mediated recruitment of GRK3. Two small molecule allosteric ligands displayed partial agonism in recruiting β-arrestins 1 and 2 and GRK-2, -3, and-5 (Compound 2 and BETP). The remaining ligands assessed (Boc5, BMS21 and TT15) were all unable to recruit regulatory proteins to the receptor, at least in this cell system. Combining previously published work performed in ChoFlpIn cells with the work presented in this thesis, we can generate a web of bias that emphasises the varying degrees of differential bias between different GLP-1R agonists (Figure 7.1-7.2). To generate this web, the operational model of agonism was fitted to the concentration response curves generated results chapters 5 and 6, to calculate a transduction ratio (tau/Ka)(Kenakin et al. 2012) for each ligand in each individual pathway. These were then normalized to a reference ligand (GLP-1(7-36)NH_2) and a reference pathway (cAMP accumulation) and then all the results plotted on the web on a log scale. The webs of bias allows a visual comparison of the signalling bias of distinct ligands and thereby distinguish different agonism profiles of GLP-1R agonists investigated in this thesis.

Web of Agonism

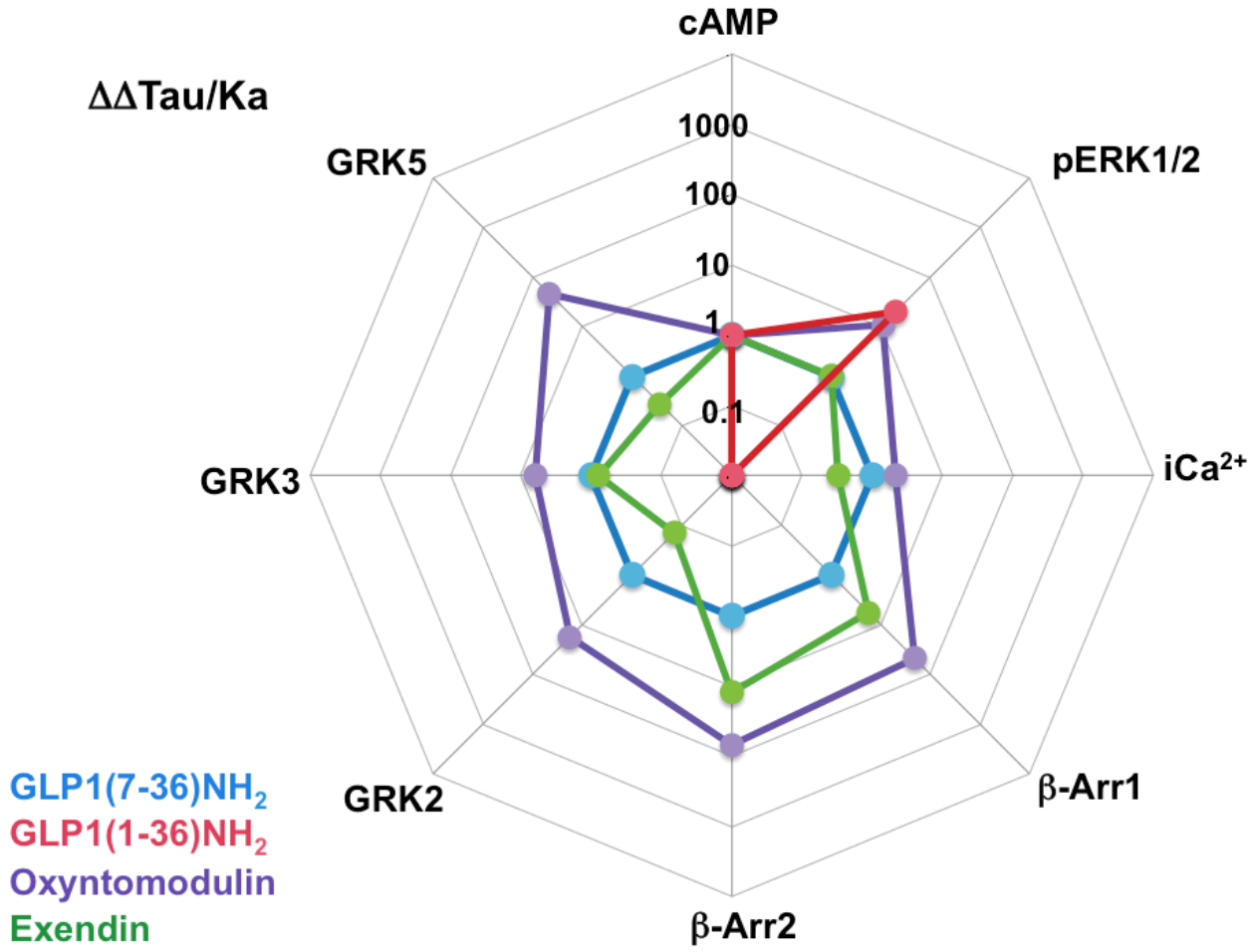

Figure 7.1. Web of bias for GLP-1R peptide agonists relative to GLP-1(7–36)NH2.

Quantification of signal bias using experimental measures of agonist affinity (Ka) and efficacy (tau) for 8 signalling pathways. The tau to Ka ratio extracted from standard concentration-response data is used to calculate bias factors or ΔΔ (tau/Ka) values through normalization of the transduction coefficient (tau/Ka) to a reference ligand and a reference pathway(Kenakin & Christopoulos 2012). All ligands display signalling bias relative to the reference agonist [GLP-1(7–36)NH_2 (blue)] and the reference pathway (cAMP). Note: log scale.

Web of Agonism

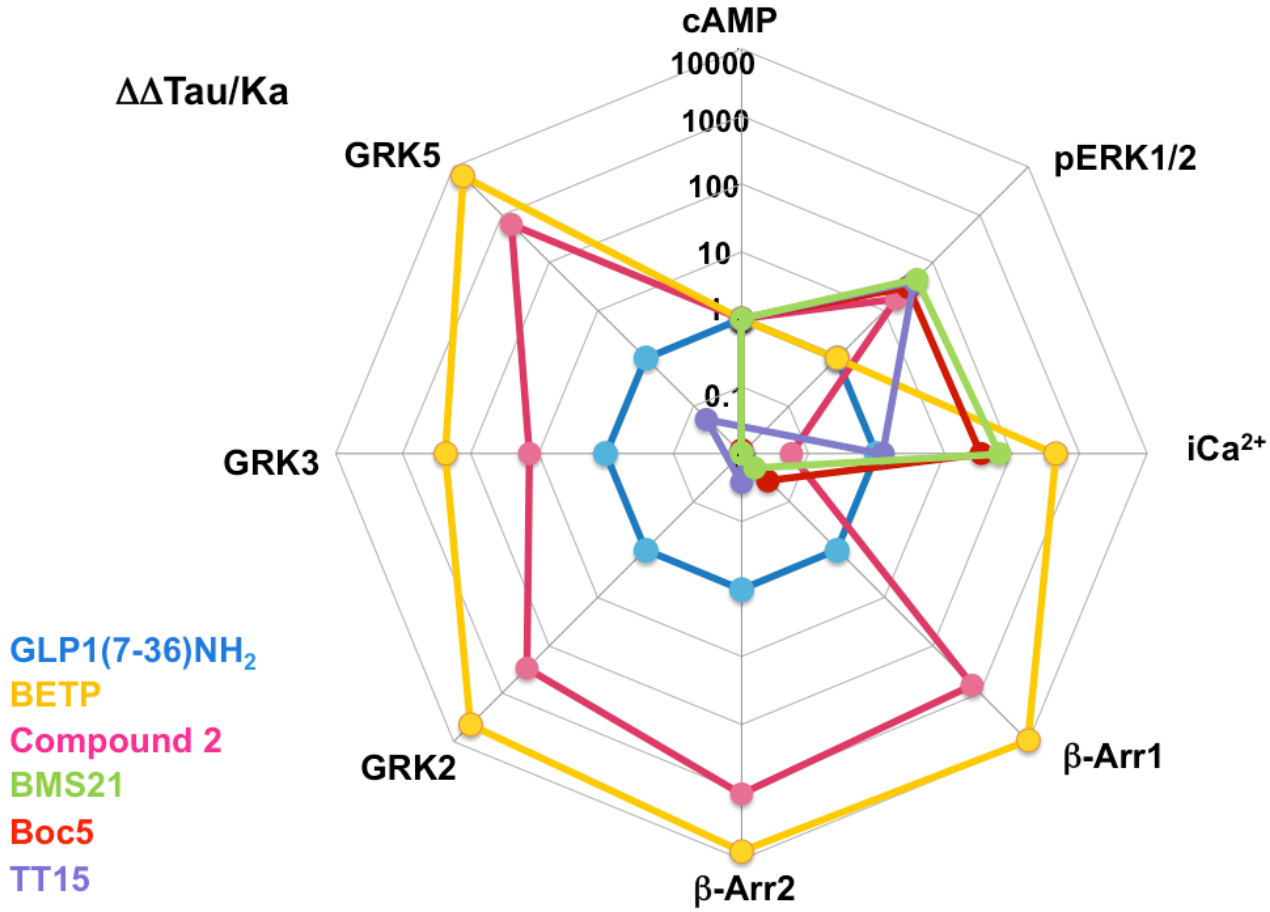

Figure 7.2. Web of bias for GLP-1R small molecule compounds relative to peptide GLP-1(7–36)NH2.

Quantification of signal bias using experimental measures of agonist affinity (Ka) and efficacy (tau) for 8 signalling pathways. The tau to Ka ratio extracted from standard concentration-response data is used to calculate bias factors or ΔΔ (tau/Ka) values through normalization of the transduction coefficient (tau/Ka) to a reference ligand and a reference pathway(Kenakin & Christopoulos 2012). All ligands display signalling bias relative to the reference agonist [GLP-1(7–36)NH_2 (blue)] and the reference pathway (cAMP). Note: log scale.

In figure 7.1, orthosteric peptide ligands are compared. Firstly, we can observe that in comparison to the primary endogenous peptide GLP-1(7-36)NH_2, the clinically used peptide exendin-4 shows a similar bias when comparing canonical signalling pathways (cAMP, Ca^{2+} mobilization and pERK1/2). However, when these studies are extended to include the regulatory proteins, differences between the bias of the two ligands begin to emerge. Exendin-4 is less biased towards GRK5 and GRK2 recruitment, however displays stronger bias towards recruitment of β-arrestin 1 and β-arrestin 2 compared to GLP-1(7-36)NH_2. In comparison, the other endogenous peptide oxyntomodulin produces a markedly different signalling profile to these two orthosteric peptides. As reported previously, while oxyntomodulin has a similar bias in cAMP accumulation and Ca^{2+} mobilization to GLP-1(7-36)NH_2 and exendin-4, it is significantly more biased than these ligands towards pERK1/2 signalling (Koole et al 2010). However, it is also significantly more biased towards recruitment of all the regulatory proteins GRK-2, -3 and -5, β-arrestin 1 and 2. This means that for the same amount of cAMP, oxyntomodulin recruits more regulatory proteins than GLP-1(7-36)NH_2 and exendin-4. In addition, although we could not detect activation of regulatory signalling pathways with GLP-1(1-36)NH_2, this ligand, as reported previously in Koole et al 2010, displayed bias towards pERK1/2 in comparison to the reference ligand GLP-1(7-36)NH_2.

Comparing the signalling bias of the synthetic small molecule ligands revealed a more striking pattern of bias than that observed with the peptide ligands (figure 7.2). This is not necessarily surprising as these ligands have a smaller pharmacophore. In addition, Compound 2 and BETP bind to a distinct site, and although the other compounds (Boc5, BMS21 and TT15), may bind to some extent bind to the orthosteric site (or sites overlapping the orthosteric site), they cannot fully recapitulate the peptide ligands due to their much smaller size. Interestingly, both Compound 2 and BETP that have an allosteric mode of action, display biased signalling towards

GRK-2, -3, and -5, β-arrestin1 and β-arrestin2 in comparison to GLP-1(7-36)NH_2, albeit that Compound 2 was weaker at recruiting these regulatory proteins compared to BETP. However, the other small molecule ligands, that are believed to at least partially overlap with orthosteric ligand binding pocket (and can be allosterically modulated by Compound 2 and BETP (Wootten et al. 2012) are biased away from recruitment of regulatory proteins. Despite the allosteric ligands Compound 2 and BETP having a similar profile with the ability to more readily recruit regulatory proteins, the biased profiles of these compounds do differ slightly. Compound 2 displayed bias towards pERK1/2 but had no agonism towards Ca^{2+} mobilization. In contrast, BETP displayed weak bias towards pERK1/2 signalling and was more biased towards Ca^{2+} mobilization. As a note, although in our system Ca^{2+} signalling could not be detected by Compound 2 at the concentrations tested, a separate study has reported weak Ca^{2+} mobilization mediated by Compound 2 via the GLP-1R in a HEK293 cell (Coopman et al. 2010). As noted above, this may imply that the signalling bias of different ligands may be different in a HEK cell background compared to a CHO cell background, or that the receptor expression the HEK system explored in (Coopman et al. 2010) is higher than in the CHO cells used in our studies, allowing the detection of weak signalling to the Ca^{2+} mobilization pathway.

Despite this thesis demonstrating bias between different ligands and signalling regulatory profiles, this bias may be cell background-dependent. However, this work identifies variances in signalling between different ligands and gives us a fingerprint for the ligands assessed. Thus we can potentially use these webs to attempt to relate these fingerprints to physiological effects in future studies to try to understand the ideal overall efficacy required to give therapeutically beneficial outcomes from GLP-1R activation, while avoiding detrimental effects. Although these webs of bias do not define absolute bias, they provide us with a simple system for which we can isolate out ligands that have different bias patterns and can then endeavor to relate our

observations from a simple system to what might be physiologically relevant, which can perhaps be further linked to screening in the future, to attempt to improve translation of hits *in vitro* to therapeutic application.

Many questions arise from interesting observations in these webs of bias that require further studies to answer. For example, there are multiple papers that have emerged recently in the literature suggesting β-arrestin 1 is linked to insulin secretion (Sonoda et al. 2008; Quoyer et al. 2010). However, all the small ligands used in the study are reported to promote insulin secretion (Su, He, Li, Liu, J. Wang, Y. Wang, W. Gao, Zhou, Liao & Young 2008a; Gigoux & Fourmy 2013; Lorenz et al. 2013; Ge et al. 2013), yet three of them (Boc5, TT15 and BMS21) are unable to recruit regulatory proteins. It is unclear at this stage, if β-arrestin1 that is recruited to the GLP-1R is responsible for promoting insulin secretion, or if it is via a different mechanism (for example, β-arrestin1 activated indirectly downstream of GLP-1R activation). The overall signalling mediated downstream by the GLP-1R in a pancreatic β cell is clearly complex and certainly involves an intricate network of multiple signalling events that lead to insulin secretion and β cell survival (see chapter 1; section 2 and 3). It may be that it is not just important what pathways are activated at the exclusion of others, but how much of one pathway is activated compared to another. Having the availability of a range of ligands with very distinct biases such as the sets of ligands explored here and being able to analyse them with webs of bias as we have shown here allows easy comparison of how much of one pathway is activated relative to an equivalent amount of the reference pathway (in this case cAMP) for each ligand relative to another. With these types of analyses, we can begin to identify tool compounds that may allow us to begin to understand the ideal interplay of different signalling outputs to give the best, most physiologically relevant overall efficacy for designing and identifying better drug compounds that are less likely to fail in clinical trials.

This thesis also explored modulation of the recruitment of regulatory proteins to the GLP-1R by allosteric ligands. Using BETP and Compound 2 in the presence of different orthosteric ligands, there was a plethora of distinct outcomes with both GRK and β-arrestin regulatory proteins. Co-addition of Compound 2 with GLP-1(7-36)NH_2, exendin-4 or oxyntomodulin mediated enhanced recruitment of β-arrestin1 and β-arrestin2. Interestingly however, GLP-1(7-36)NH_2 and oxyntomodulin in combination with Compound 2 mediated enhanced recruitment of GRK2, whereas compound 2 positively modulated exendin-mediated recruitment of GRK3. In contrast, BETP only modulated exendin-4 mediated recruitment of GRK3 without influencing β-arrestin recruitment. The physiological relevance of these effects are difficult to predict at this stage, thus future studies are required to distinguish the effects of different regulatory proteins and their effects on downstream signalling. As noted, to date, there is strong evidence that both GLP-1R-mediated Ca^{2+} mobilization and cAMP accumulation are linked to insulin secretion in islets (Selway et al. 2012; Wootten et al. 2013; Willard & Sloop 2012; Miura & Matsui 2003; Tsuboi et al. 2003), while β-arrestin1-mediated pERK1/2 signalling is associated with β-cell survival (Sonoda et al. 2008). Moreover, Ca^{2+}-mediated pERK1/2 via Ca^{2+} influx from L-type voltage gated Ca^{2+} channels can also occur although whether this is specifically linked to survival is unknown (Selway et al. 2012). This picture presents a very limited view of the complexity of this system for which many endogenous ligands exist. The findings from this thesis open up many questions with regards to the full signalling repertoire of regulatory proteins and whether specific activation of GRKs and/or β-arrestins in response to distinct ligands can mediate a more physiological beneficial outcome in comparison to others with respect to T2D treatment. To date, there has been no link identified as to the physiological relevance of GLP-1R mediated β-arrestin2 recruitment, or indeed the importance of distinct GRKs in this system. β-arrestin2 has been reported to play an important role in insulin docking at the cell membrane (M. Zhang et al.

2013a). In addition, studies have identified that specific domains of endogenous GRK2 function as negative regulators of insulin-stimulated glucose transport by interfering with Gαq/11 signalling-mediated GLUT4 translocation, suggesting that inhibitors of GRK2 may lead to enhanced insulin sensitivity (Usui et al. 2004). Therefore these aspects of signalling need to be explored in more detail in context of the activation of the GLP-1R as the ability of distinct ligands to promote different signalling profiles with respect to these proteins may have therapeutic implications.

Summarised in figure 7.3 is the culmination of modulation data from this thesis combined with data that has been previously published from the Sexton laboratory. This figure highlights the significant effects of allosteric modulation and probe-dependence in GLP-1R signalling and signal bias between different orthosteric ligands. From this, it is evident that very few allosteric-orthosteric ligand combinations display a similar level of effect in their signalling outputs. In particular this schematic emphasises that in comparison to BETP, Compound 2 induces significant positive modulation in the recruitment of the regulatory proteins β-arrestin1 and β-arrestin2 by the three higher affinity peptide ligands (panels a, b and c). However probe-dependence is still evident here with the exact signalling output dependent on the GLP-1R orthosteric ligand co-bound. In the presence of Compound 2, the GLP-1(7-36)NH_2 -mediated signal profile is biased towards both β-arrestins and GRK3 recruitment, whereas the exendin-4-mediated signalling profile displays bias towards β-arrestins and GRK2 (a, b). Probe dependence is furthermore displayed in the presence of oxyntomodulin and Compound 2, whereby both β-arrestins and GRK3 were significantly modulated by Compound 2, with additional potentiation of the cAMP signalling pathway (c). Interestingly, co-addition of metabolite GLP-

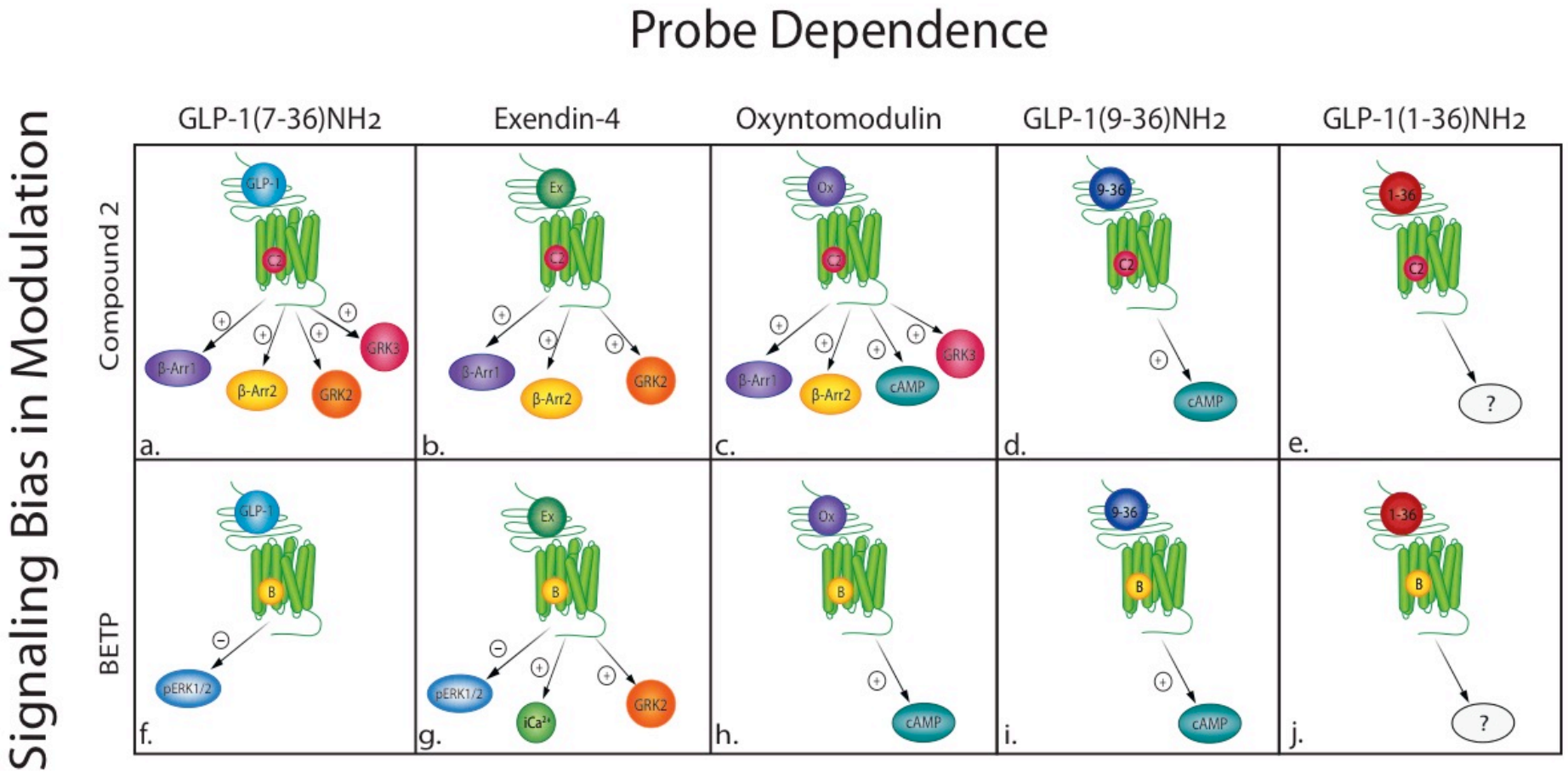

Figure 7.3. **Schematic of signalling bias in modulation induced by small allosteric molecule Compound 2 and BETP**;
a, f displays bias between Compound 2 and BETP on GLP-1(7-36)NH_2; b, g displays bias between Compound 2 and BETP on exendin-4; c, h show the bias between Compound 2 and BETP on oxyntomodulin, panels d, i, display the bias between Compound 2 and BETP on metabolite GLP-1(9-36)NH_2. Panels a, b, c and d represent probe dependence by Compound 2, where panels f, g, h and i, display probe dependence by BETP. There was no probe dependence or bias signalling between GLP-1(1-36)NH_2 and both small molecules Compound 2 and BETP (e, j). This figure displays statistically significant $p < 0.05$ modulated signalling pathways mediated by small allosteric molecules when co-bound GLP-1R peptide ligands.

1(9-36)NH_2 and Compound 2 did not show any potentiation of regulatory proteins, but modulated cAMP signalling (d) (as discussed above), and within the pathways assessed, no potentiation of any signalling was observed for GLP-1(1-36)NH_2.

Investigation of the allosteric modulator BETP revealed evidence of distinct modulation signalling profiles of the orthosteric peptides. In contrast to Compound 2, BETP appears to steer the peptide mediated signal in a different direction, enhancing/reducing other intracellular pathways such as pERK1/2, iCa^{2+}, cAMP and GRK2 recruitment with no effect on arrestin recruitment. For both GLP-1(7-36)NH_2 and exendin-4, co-addition of BETP resulted in negative modulation of pERK1/2 (f, g), but exclusive to exendin-4 there was also enhanced signalling of iCa^{2+} mobilization and GRK2 recruitment (g). Despite oxyntomodulin displaying a wide repertoire of positive modulation with Compound 2, BETP only positively modulated oxyntomodulin-mediated cAMP accumulation (h). This is particularly interesting as, like Compound 2, BETP potentiates the affinity of oxyntomodulin at the GLP-1R, so the lack of modulation in all pathways except cAMP, suggests that perhaps there is negative modulation of signalling efficacy, resulting in a net effect (from affinity and efficacy) of neutral modulation down these pathways. The metabolite GLP-1(9-36)NH_2 and BETP displayed a similar profile to that seen with Compound 2, in that there was only positive modulation of cAMP signalling (i) and as with Compound 2, there was no observed modulation of any pathway with GLP-1(1-36)NH_2 and BETP.

These studies clearly highlight that even orthosteric ligands that may appear similar in their functional properties (ie GLP-1(7-36)NH2 and exendin-4), can display very different profiles when combined with allosteric ligands. Further work is required in order to understand the impact of modulating specific intracellular proteins on β-cell function. For three of the peptides

highlighted in the figure, there is *in vivo* data on insulin secretion generated from collaborative efforts (Sloop and Willard at Eli Lily). Modulation of GLP-1(7-36)NH_2 by small molecules results in no enhancement of blood insulin levels as determined by an IVGTT (Willard, Wootten, et al. 2012b). As noted above, GLP-1(9-36)NH_2 mediated insulin secretion can be induced by co addition of BETP *in vivo*. Furthermore, modulation of oxyntomodulin by BETP can also enhance blood insulin levels (Willard, Wootten, et al. 2012b) (appendix 1). In our system (CHO cells), both of these ligands only enhance cAMP pathway when co-activated by BETP (Wootten et al. 2012; Willard, Wootten, et al. 2012b). This suggests that perhaps cAMP modulation alone is sufficient as an improved therapeutic output, at least in the context of un-modulated signalling via these pathways. It is interesting to note that Compound 2 modulates additional regulatory pathways when bound with oxyntomodulin, and therefore this would be interesting to explore further to see if this provides additional therapeutic benefit. However, due to the strong agonism of Compound 2 (for cAMP and insulin secretion), it is difficult to assess and interpret the findings of this ligand *in vivo*. Future work may include identifying a range of further compounds that display minimal agonism but induce differential bias in peptide signal outputs that may aid in elucidating and answering these questions.

Although not included in this thesis, preliminary experiments have begun using multiple inhibitors and siRNA knockdown studies in attempt to explore the importance of distinct signalling and regulatory proteins and the interplay of these pathways in physiologically important endpoints such as cell survival and insulin release. This future work will provide insight into the physiological importance of specific regulatory proteins and how they contribute to β-cell function and the interplay of signalling from regulatory proteins and classical signalling pathways. Through the exploration of biased ligands and biased modulators in these systems, we

may also begin to translate our findings from this thesis into physiological effects and thus this work may assist in future therapeutic drug design.

Throughout this thesis the main focus for discussion has been on translation from *in vitro* systems into islets, as the GLP-1R is a major therapeutic target for T2D. Further areas of investigation into areas that will be of benefit for treating T2D requires exploration around the effect of allosteric modulation and pathways linked to activation of other physiologically relevant endpoints. This could potentially include pathways associated with β cell survival and function, gastric emptying and satiety. Additionally, the GLP-1R is expressed in the brain and cardiomyocytes, and thus has also been implicated as a therapeutic target for cardiovascular diseases, Alzheimer's and Parkinson's disease (Y. Li et al. 2010; Burgmaier et al. 2012; Y. Li et al. 2009; Y. Li et al. 2012b). Therefore, extending these studies outside islet biology to neurons and cardiomyocytes may aid in drug development for other diseases states. It would be of interest to explore whether there are changes in the nature of the signalling bias, as the bias in different tissues might be altered, therefore linking what a fingerprint might look like in different tissues and cell types may potentially provide selective therapeutics targeting the same receptor for different disease states.

This thesis has extended previous knowledge around biased signalling and allosteric modulation and has also developed a new higher throughput system to use BRET to study GRK and β-arrestin interactions. Although biased signalling and allosteric modulation (particularly their probe dependent nature for modulation of different endogenous ligands) are now documented at the GLP-1R, the physiological significance of these phenomenon still remains largely unknown. The challenge in the field now moving forward is to translate the findings around bias and allosteric modulation into physiological systems to understand the therapeutic implications. The tools and concepts developed in this thesis are a step towards beginning to understand GLP-1R

function, and will address challenges associated with discovery, validation and development of novel, selective drugs with an improved therapeutic profile for the management of T2D.

CHAPTER 8:

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APPENDIX 1

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

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.

Mol Pharmacol **82:**1066–1073, 2012

1521-0111/12/8206-1066–1073$25.00
MOLECULAR PHARMACOLOGY
Vol. 82, No. 6
80432/3804157

Mol Pharmacol 82:1066–1073, 2012

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

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

Translational Science and Technologies (F.S.W.), Endocrine Discovery (A.D.S., J.F., T.B.F., K.B., K.W.S.), and BioTechnology Discovery Research (J.A.-F.), Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana; and Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria, Australia (D.W., E.E.S., S.G.B.F., A.C., P.M.S.)

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-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 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-translational 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-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; HEK, human embryonic kidney; CHO, Chinese hamster ovary; Rluc, *Renilla* luciferase; BRET, bioluminescence resonance energy transfer; GTPγS, 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_2/(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_2/(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_2/(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_2/(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_2/(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_2/(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_2/(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_2/(7–37) is 1 to 2 min (Siegel et al., 1999), whereas half-life estimates for oxyntomodulin range from 6 to 12 min (Baldissera 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_2 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_2 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 μg/ml streptomycin, and 20 mM HEPES. Cells were transfected in suspension at 6.25×10^4 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 μM 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-μl 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, Flp-In CHO cells expressing the human GLP-1 receptor at a density of 120,000 receptors/cell were used; intracellular Ca^{2+} mobilization, ERK1/2 phosphorylation, and cAMP accumulation were measured as described previously (Wootten et al., 2012).

***β*-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 β-arrestin1- or β-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 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 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, 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 did not alter its pharmacology as assessed in cAMP accumulation, phosphorylated 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 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 μM. 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 β-arrestin1 and β-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}S]GTPγS and ^{125}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 $MgCl_2$, and 1× 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 1800*g* 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 25,000*g* 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}S]GTPγS binding to $G\alpha_s$ using an antibody capture scintillation proximity assay (DeLapp et al., 1999). Reactions contained 50 μg of 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-labeled 500 pM guanosine 5′-(γ-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 μg/ml rabbit anti-$G\alpha_{s/olf}$ 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 80*g* 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}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):

$$Y = \frac{B_{\max} \times [A]}{[A] + K_{\mathrm{App}}} + \mathrm{NS} \tag{1}$$

where

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

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. 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 $\alpha > 1$ 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):

$$E = \frac{E_{\mathrm{m}}(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + [B]K_A + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \tag{3}$$

$$E = \frac{E_{\mathrm{m}}(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]\mathrm{EC}_{50})^n}{\mathrm{EC}_{50}{}^n(K_B + [B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \alpha\beta[B]) + \tau_B[B]\mathrm{EC}_{50})^n} \tag{4}$$

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, 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 an allosteric ligand (BETP) and a partial agonist (intracellular Ca^{2+}, β-arrestin1, and β-arrestin2), whereas eq. 4 was used when BETP interacted with a full agonist (GTPγS, 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 ± 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_2 but shows little modulation of the active, circulating forms of GLP-1, GLP-1(7–36)-NH_2/(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_2/(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_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 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 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) (Fig. 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 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 ^{125}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 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 Gα_s-specific GTPγS 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_2 signal is approximately 12,000 cpm (Supplemental Fig. 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 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_2 (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_2. BETP alone is a low-potency partial agonist in the GTPγS binding

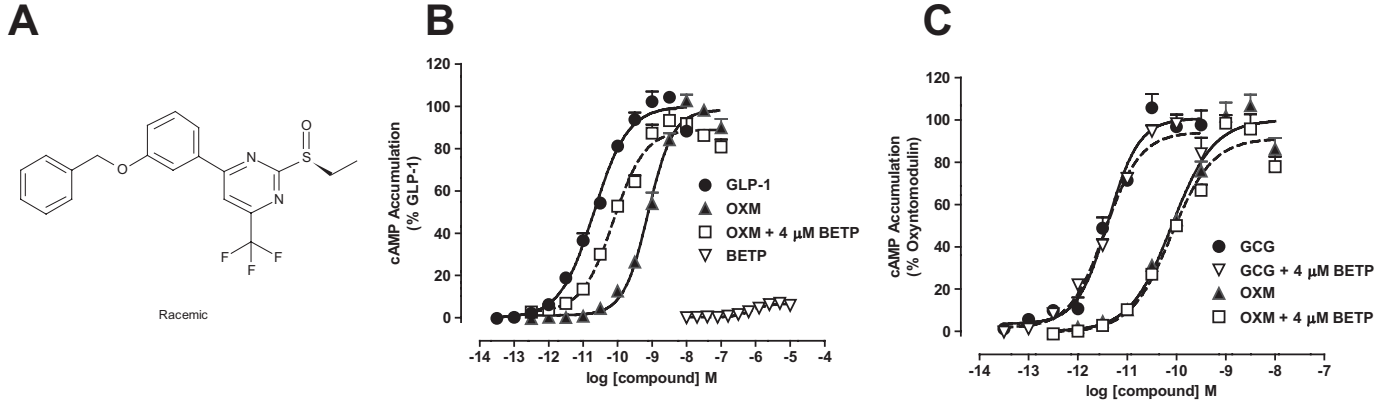

Fig. 1. Oxyntomodulin-stimulated GLP-1 receptor signaling is specifically enhanced by a small molecule GLP-1 receptor allosteric modulator. A, the chemical structure of the low-molecular-weight GLP-1 receptor modulator, BETP, used in these studies. B, ligand-stimulated cAMP production is measured in HEK293 cells expressing the human GLP-1 receptor. Concentration-response curves are depicted for oxyntomodulin (OXM) in the absence or presence of 4 μM BETP. Data are fit to the four-parameter logistic equation and calculated EC_{50} and E_{max} values [percentage 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%; and 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 (percentage of maximal OXM effect) are as follows: GCG, 4 pM, 101%; GCG + 4 μM BETP, 4 pM, 94%; OXM, 80 pM, 100%; and 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.

assay entirely consistent with its pharmacological profile in cAMP accumulation assays (Supplemental Fig. 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 Fig. 3).

BETP Enhances Oxyntomodulin-Induced Insulin Secretion. To test whether 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 (Fig. 3). Here, infusion of GLP-1(7–36)-NH_2 stimulates an acute insulinotropic response upon coadministration of a glucose bolus, consistent with the known biology of this peptide (Holst, 2007). Likewise, oxyntomodulin dose-dependently induces insulin secretion and at saturating concentrations provides efficacy equivalent 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

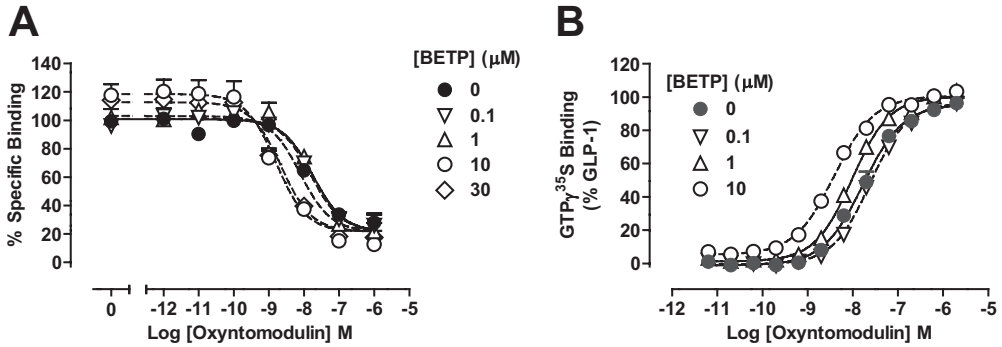

Fig. 2. BETP increases the binding affinity of oxyntomodulin for the GLP-1 receptor and enhances activation of the $G\alpha_s$ heterotrimeric G protein. A, positive binding cooperativity of BETP and oxyntomodulin is demonstrated in competition binding studies using GLP-1 receptor-expressing membranes. The potency of oxyntomodulin to displace specific binding of ^{125}I-exendin-4(9–39) is measured in the presence of fixed concentrations of BETP. B, oxyntomodulin-mediated [^{35}S]GTPγS binding to endogenous $G\alpha_s$ protein in GLP-1 receptor-expressing membranes is determined by antibody capture scintillation proximity. The potency of oxyntomodulin 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; the resultant calculated parameters are reported in Table 1.

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 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 (α) and efficacy (β) modulation by the allosteric ligand. This is calculated using an operational model of agonism as defined in eqs. 3 and 4. pIC_{50}, pEC_{50}, and E_{max} values for these data sets are presented in Supplemental Table 1.

Assay Measurement	Logα (α)	Log$\alpha\beta$ ($\alpha\beta$)	Logβ (β) (Log$\alpha\beta$ − Logα)
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)
Phospho-ERK1/2		−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-Arrestin1		0.40 ± 0.08 (2.51)	−0.76 ± 0.13 (0.17)
β-Arrestin2		0.54 ± 0.19 (3.47)	−0.62 ± 0.21 (0.24)

*Statistically significant at $p < 0.05$.
[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.

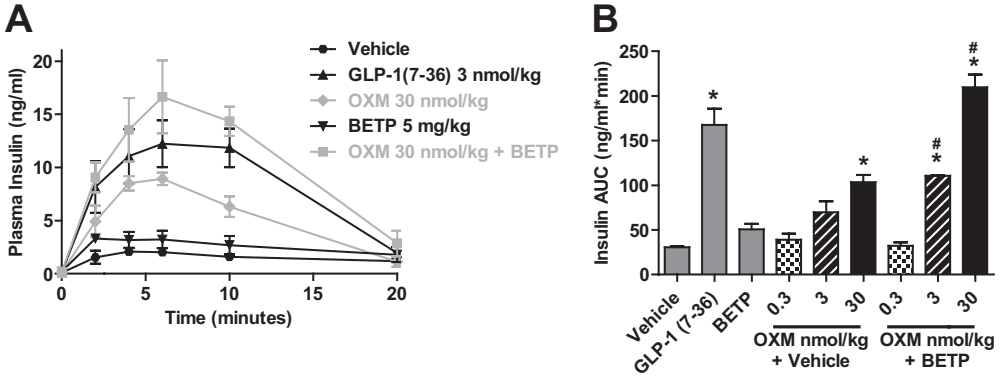

Fig. 3. Oxyntomodulin-stimulated insulin secretion is enhanced by BETP in vivo. A, time course of plasma insulin concentrations in fasted, anesthetized Wistar rats treated with either vehicle, GLP-1(7–36)-NH_2 (3 nmol/kg), oxyntomodulin (OXM) (30 nmol/kg), BETP (5 mg/kg), or OXM (30 nmol/kg) + BETP (5 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 ± S.E.M. is as follows: BETP (5 mg/kg), 20 ± 6 ng/ml · min; oxyntomodulin (30 nmol/kg), 73 ± 8 ng/ml · min; and BETP (5 mg/kg) + oxyntomodulin (30 nmol/kg), 179 ± 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_2 with varying degrees of positive and negative cooperativity for cAMP accumulation, ERK1/2 activation, and Ca^{2+} mobilization pathways (Wootten 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^{2+} mobilization (Fig. 4B; Table 1) and efficacy-driven negative cooperativity for ERK1/2 activation (β = 0.03) are observed (Fig. 4C; Table 1). The other major reported pathway of GLP-1 receptor signaling is via β-arrestin (Jorgensen et al., 2005). We measured oxyntomodulin-induced β-arrestin1 recruitment using BRET (Fig. 4D). Oxyntomodulin is a potent agonist of β-arrestin1 recruitment with potency and efficacy equivalent to those of 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 that 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 that 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 Fig. 4). These data therefore indicate that BETP allosteric modulation of oxyntomodulin in heterologous systems is strongly biased toward cAMP accumulation and neutral toward β-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_2/(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 DDP-4 inhibitor class because the GLP-1 analogs elicit larger percentage hemoglobulin A_{1c} 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_2/(7–37) (DeFronzo et al., 2008). In contrast, treatment with the DPP-4 inhibitor, sitagliptin,

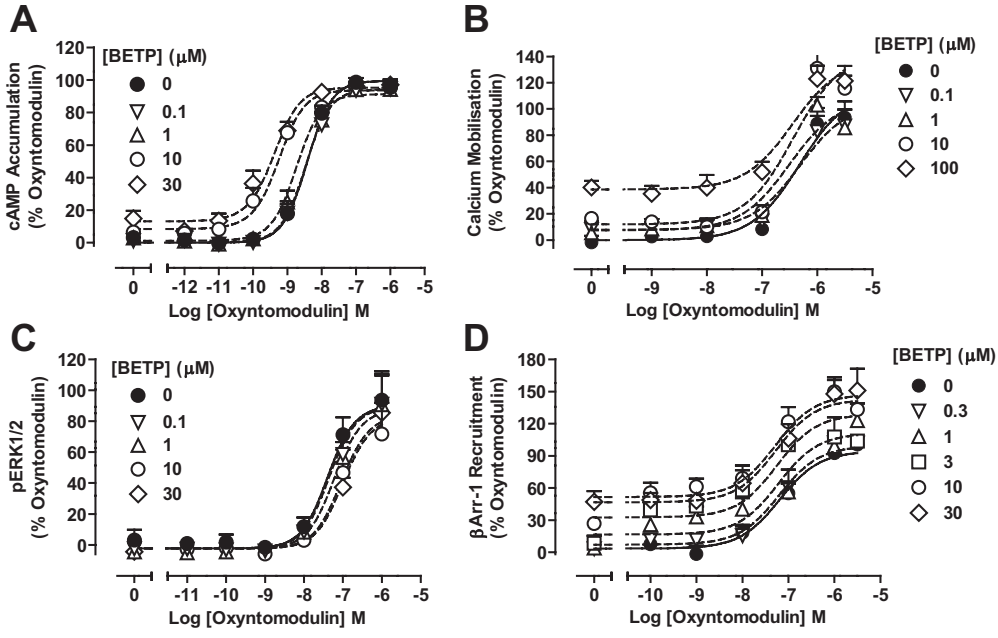

Fig. 4. BETP induces cAMP biased signaling at the oxyntomodulin-bound GLP-1 receptor. The dose-dependent effects of BETP on oxyntomodulin-mediated activation of (A) cAMP accumulation, (B) intracellular Ca^{2+} mobilization, (C) ERK1/2 phosphorylation, and (D) β-arrestin recruitment are quantified in 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_2/(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_2/(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_2/(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_2/(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 $G\alpha_s$ 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 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^{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 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_2/(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_2, but not the parent agonist GLP-1(7–36)-NH_2 (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_2, 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 GTPγS 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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Address correspondence to: Dr. Kyle W. Sloop, Endocrine Discovery, Lilly Research Laboratories, Drop Code 0424, Indianapolis, IN 46285. [redacted]

APPENDIX 2

Differential Activation and Modulation of the Glucagon-Like Peptide-1 Receptor by Small Molecule Ligands

Denise Wootten, Emilia E. Savage, Francis S. Willard, Ana B. Bueno, Kyle W. Sloop, Arthur Christopoulos, and Patrick M. Sexton.

Mol Pharmacol **83:** 822–834

1521-0111/83/4/822–834$25.00
MOLECULAR PHARMACOLOGY

http://dx.doi.org/10.1124/mol.112.084525
Mol Pharmacol 83:822–834, April 2013

Differential Activation and Modulation of the Glucagon-Like Peptide-1 Receptor by Small Molecule Ligands[S]

Denise Wootten, Emilia E. Savage, Francis S. Willard, Ana B. Bueno, Kyle W. Sloop, Arthur Christopoulos, and Patrick M. Sexton

Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Parkville, Victoria, Australia (D.W., E.E.S., A.C., P.M.S.); Endocrine Discovery (K.W.S.) and Translational Science and Technologies (F.S.W.), Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana; Centro de Investigación Lilly, Eli Lilly and Company, Alcobendas, Spain (A.B.B.)

Received December 17, 2012; accepted January 24, 2013

ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is a major therapeutic target for the treatment of type 2 diabetes due to its role in glucose homeostasis. Despite the availability of peptide-based GLP-1R drugs for treatment of this disease, there is great interest in developing small molecules that can be administered orally. The GLP-1R system is complex, with multiple endogenous and clinically used peptide ligands that exhibit different signaling biases at this receptor. This study revealed that small molecule ligands acting at this receptor are differentially biased to peptide ligands and also from each other with respect to the signaling pathways that they activate. Furthermore, allosteric small molecule ligands were also able to induce bias in signaling mediated by orthosteric ligands. This was dependent on both the orthosteric and allosteric ligand as no two allosteric-orthosteric ligand pairs could induce the same signaling profile. We highlight the need to profile compounds across multiple signaling pathways and in combination with multiple orthosteric ligands in systems such as the GLP-1R where more than one endogenous ligand exists. In the context of pleiotropical coupling of receptors and the interplay of multiple pathways leading to physiologic responses, profiling of small molecules in this manner may lead to a better understanding of the physiologic consequences of biased signaling at this receptor. This could enable the design and development of improved therapeutics that have the ability to fine-tune receptor signaling, leading to beneficial therapeutic outcomes while reducing side effect profiles.

Introduction

Type 2 diabetes mellitus and its associated obesity are predominantly characterized by a decrease in peripheral tissue response to insulin in association with impaired pancreatic β-cell function that results in an increase in fasting glycemia (DeFronzo, 1992). The incretin hormone, glucagon-like peptide-1 (GLP-1) has well established effects on pancreatic β-cell insulin secretion and, despite a reduction in secreted levels of this hormone in diabetic patients, it retains its potent insulinotropic activity. This action combined with a number of other important effects, including reduction in glucagon secretion, delayed gastric emptying, induction of satiety, and increasing pancreatic β-cell mass, have attracted significant interest in GLP-1 and related analogs for the treatment of type 2 diabetes mellitus (Drucker and Nauck, 2006).

GLP-1 exerts its effects by binding to the GLP-1 receptor (GLP-1R), which belongs to the family B subclass of the G protein-coupled receptor (GPCR) superfamily. In recent years, it has become clear that individual GPCRs can exist in multiple receptor conformations and can elicit numerous functional responses, both G protein- and non-G protein-mediated. This has led to the discovery that different ligands can stabilize distinct subsets of receptor conformations that can "traffic" stimulus to diverse functional outputs with varying prominence, a concept referred to as biased agonism (also known as functional selectivity, stimulus bias or ligand-directed signaling) (Kenakin, 2011). The GLP-1R is predominantly expressed in pancreatic β-cells and mediates its effects through coupling primarily to $G\alpha_s$, resulting in an increase in cAMP, cell depolarization and an increase in

This work was funded by National Health and Medical Research Council project [Grants 1002180 and 519461].
dx.doi.org/10.1124/mol.112.084525.
[S] This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: β-Arr, beta arrestin; BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; Boc5, 1,3-bis [[4-(tert-butoxycarbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CHO, Chinese hamster ovary; Compound 2, 6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; ERK1/2, extracellular signal-related kinases 1 and 2; FBS, fetal bovine serum; Forskolin, (3R,4aR,5S,6S,6aS,10S,10aR,10bS)-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-3-vinyldodecahydro-1H-benzo[f]chromen-5-yl acetate; GLP-1, glucagon-like peptide; GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein-coupled receptor; iCa^{2+}, intracellular calcium; TT15, (2S)-2-[[(8S)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid.

cytosolic calcium that ultimately promotes insulin secretion (Drucker et al., 1987; Holz et al., 1993). Although cAMP formation is a critical component of GLP-1R-mediated signaling required for insulin secretion, there are also roles of other signaling pathways in augmentation of insulin responses. In addition to cAMP formation, activated GLP-1Rs can promote epidermal growth factor receptor transactivation (Buteau et al., 2003), phosphatidylinositol 3 kinase activity, insulin receptor substrate-2 signaling (Park et al., 2006), extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity (Montrose-Rafizadeh et al., 1999), mobilization of intracellular calcium (iCa^{2+}) (Baggio and Drucker, 2007), as well as nuclear translocation of protein kinase C to mediate β-cell proliferation and differentiation and promote insulin gene transcription (Buteau et al., 2003). Recent studies also support an essential role of β-arrestins in downstream GLP-1R-mediated insulin secretion (Sonoda et al., 2008; Quoyer et al., 2010). Although some of these pathways have been linked to therapeutically relevant outputs, such as insulin secretion and β-cell survival, the underlying GLP-1R-mediated signaling required for therapeutically beneficial effects, such as delaying gastric emptying and inducing satiety, are not fully understood.

Currently, approved therapeutics acting at the GLP-1R are peptide-based; however, there is substantial interest in development of small molecule drugs. In recent years, an increasing number of reports have shown discovery of structurally diverse small molecule agonists of the GLP-1R (Willard et al., 2012a). These include (but are not limited to) a series of quinoxalines, the best characterized being Compound 2 (6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline), a series of pyrimidines, the best characterized being BETP (4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine), substituted cyclobutanes such as Boc5 (1,3-bis [[4-(tert-butoxy-carbonylamino) benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid), and a series of compounds reported in patents by Transtech Pharma. In addition to displaying agonism in their own right, small molecule compounds that bind allosterically to the GLP-1R have the potential to modulate the function of endogenous hormones, allowing fine control of receptor function and/or spatial and temporal elements of endogenous orthosteric peptide signaling. There are many orthosteric peptide agonists of the GLP-1R, including multiple endogenous ligands, as well as several peptides that are used therapeutically or are in clinical trials (Baggio and Drucker, 2007). All peptide agonists studied to date preferentially activate cAMP over ERK1/2 and iCa^{2+} mobilization in vitro (Koole et al., 2010). However, the relative degree of bias is variable between ligands, with truncated GLP-1 peptides and exendin-4 having greater bias toward cAMP than full-length GLP-1 peptides and oxyntomodulin (Koole et al., 2010). In addition, allosteric ligands can differentially alter the signaling profile mediated by these endogenous peptides and can therefore induce biased signaling in a peptide-specific manner.

While most of the small molecules developed to date are not drug-like compounds, they may represent pharmacophores that can be further optimized for clinical evaluation. They also provide us with a range of useful research tools that can be used to help understand the mechanism by which these small molecules bind and exert their physiologic effects. In this study, we used an analytical approach, investigating the signaling of the GLP-1R across multiple signaling pathways to assess and quantify stimulus bias for a range of low molecular weight ligands (both peptide and nonpeptide). The ability of these small ligands to act allosterically to modulate the responses and bias of distinct orthosteric peptide ligands was also assessed.

Materials and Methods

Materials. Small molecule GLP-1 ligands BETP (Sloop et al., 2010), Compound 2 (Knudsen et al., 2007), Boc5 (Chen et al., 2007), [(2*S*)-2-[[(8*S*)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid] (TT15) (Rao, 2009), and BMS21 (Mapelli et al., 2009) were synthesized according to literature and standard methods (see Supplemental Data, experimental procedure for more details). GLP-1(7–36)NH_2, GLP-1(1–36)NH_2, exendin-4, and oxyntomodulin were purchased from American Peptide Company (Sunnyvale, CA). Dulbecco's modified Eagle's medium and Fluo-4 AM were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Thermo Electron Corporation (Melbourne, VIC, Australia). AlphaScreen reagents, 96-well UniFilter GF/C filter plates, 384-well Proxiplates, and Microscint 40 scintillant were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). SureFire ERK1/2 reagents were obtained from TGR Biosciences (Adelaide, SA, Australia). 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. Human GLP-1Rs were isogenically integrated into FlpIn-Chinese hamster ovary (Flp-In-CHO) cells (Invitrogen) and selection of receptor-expressing cells accomplished by treatment with 600 μg/ml hygromycin-B as previously described (May et al., 2007). Transfected and parental Flp-In-CHO cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS and incubated in a humidified environment at 37°C in 5% CO_2.

Radioligand Binding Assay. Flp-In-CHO GLP-1R 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, and radioligand binding carried out as previously described (Koole et al., 2011). For each cell line in all experiments, total binding was defined by 0.5 nM ^{125}I-exendin(9–39) alone, and nonspecific binding was defined by 1 μM exendin(9–39). For analysis, data are normalized to the specific binding for each individual experiment.

cAMP Accumulation Assay. Flp-In-CHO wild-type and mutant human GLP-1R 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, and cAMP detection carried out using the PerkinElmer AlphaScreen kit, as previously described (Koole et al., 2010). All values were converted to concentration of cAMP using a cAMP standard curve performed in parallel, and data were subsequently normalized to the response of 100 μM forskolin.

pERK1/2 Assay. Flp-In-CHO GLP-1R 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. Receptor-mediated pERK1/2 was determined using the AlphaScreen ERK1/2 SureFire protocol as previously described (May et al., 2007). Initial pERK1/2 time course experiments were performed over 1 hour to determine the time at which agonist-mediated pERK1/2 was maximal. Subsequent experiments were then performed at the time required to generate a maximal pERK1/2 response (7 minutes). Data were normalized to the maximal response elicited by 10% FBS determined at 6 minutes (peak FBS response).

Intracellular Ca^{2+} Mobilization Assay. Flp-In-CHO GLP-1R 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, and receptor-mediated iCa^{2+} mobilization determined as previously described (Werry et al., 2005). Fluorescence was determined immediately after ligand addition, with an excitation wavelength set to 485 nm and an emission wavelength set to 520 nm, and readings taken every 1.36 seconds for 120 seconds. Peak magnitude was calculated using five-point smoothing, followed by correction against basal fluorescence. The peak value was used to create concentration-response curves. Data were normalized to the maximal response elicited by 100 μM ATP.

β-Arrestin Recruitment Assays. Flp-In-CHO cell lines stably expressing GLP-1 receptor-Rluc8 and either β-arrestin (β-Arr)1- or β-Arr2-Venus were generated using gateway technology as previously described (Willard et al., 2012b). 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 Hanks' balanced salt solution to remove traces of phenol red and incubated in fresh Hanks' 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 bioluminescence resonance energy transfer (BRET) readings were collected using a LumiSTAR Omega instrument (BMG Labtech, Ortenberg, Germany) 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-Arr1 and β-Arr2 recruitment was maximal for each ligand in the absence and presence of BETP. Coaddition of ligands was performed for interaction assays and BRET signals were collected at this peak time point.

Data Analysis. Data were analyzed using Prism 5.03 (GraphPad, La Jolla, CA) using the three-parameter logistic equation or an operation model of allosteric agonism.

Allosteric modulator-inhibition binding data were fitted to the following allosteric-ternary complex model. In this case, nondepletion of ligands was assumed (Avlani et al., 2008):

$$Y = \frac{B_{\max} \times [A]}{[A] + K_{\mathrm{APP}}} + NS \tag{1}$$

where

$$K_{\mathrm{APP}} = \frac{K_A \times K_B}{\alpha \times [B] + K_B} \times \frac{1 + [I]/K_I + [B]/K_B + (\alpha' \times [I] \times [B])}{K_I \times K_B} \tag{2}$$

where Y represents radioligand binding, $B_{\max}$ denotes maximal binding site density, and NS denotes the fraction of nonspecific binding. $[A]$ and K_{APP} denote the concentration of radioligand and equilibrium dissociation constant for the radioligand, respectively. $[B]$ and K_{B} denote the concentration of allosteric ligand and equilibrium dissociation constant for the allosteric ligand, respectively. $[I]$ and K_{I} denote the concentration of peptide agonist used in competition with the radioligand and the equilibrium dissociation constant for the peptide agonist, respectively. α and α' represent cooperativity factors, which are defined as the allosteric interaction of the modulator with the radioligand, and modulator with the peptide agonist, respectively. Values of α or $\alpha' > 1$ are indicative of an allosteric-mediated increase in binding activity, while values of $0 < \alpha$ or $\alpha' < 1$ are indicative of an allosteric-mediated decrease in binding affinity.

To compare agonist profiles and quantify stimulus bias (functional selectivity) between the different ligands, agonist concentration-response curves were fitted to the following form of the operational model of agonism ((Black and Leff, 1983; Koole et al., 2010; Evans et al., 2011),

$$Y = basal + \frac{(E_{\mathrm{m}} - basal)\left(\frac{\tau}{K_A}\right)^n [A]^n}{[A]^n \left(\frac{\tau}{K_A}\right)^n + \left(1 + \frac{[A]}{K_A}\right)^n} \tag{3}$$

where E_{m} is the maximal possible response of the system; *basal* is the basal level of response; K_A denotes the equilibrium dissociation constant of the agonist (A); τ is an index of the signaling efficacy of the agonist and is defined as $R_{\mathrm{T}}/K_{\mathrm{E}}$, where R_{T} is the total number of receptors and K_{E} is the coupling efficiency of each agonist-occupied receptor; and n is the slope of the transducer function that links occupancy to response. The analysis assumes that the maximal system responsiveness (E_{m}) and the transduction machinery used for a given cellular pathway are the same for all agonists, such that the E_{m} and transducer slope (n) are shared between agonists. The ratio, τ/K_A (determined as a logarithm, i.e., log (τ/K_A)) is referred to herein as the "transduction coefficient" (Kenakin et al., 2012), as this composite parameter is sufficient to describe agonism and bias for a given pathway, i.e., stimulus-biased agonism can result from either a selective affinity ($K_A{}^{-1}$) of an agonist for a given receptor state(s) and/or a differential coupling efficacy (τ) toward certain pathways. To cancel the impact of cell-dependent effects on the observed agonism at each pathway, the log (τ/K_A) values were then normalized to that determined for the endogenous agonist, GLP-1(7–36)NH_2, at each pathway to yield a "normalized transduction coefficient," $\Delta\log(\tau/K_A)$, i.e., $\Delta\log(\tau/K_A) = \log(\tau/K_A)_{\mathrm{test}} - \log(\tau/K_A)_{\mathrm{GLP\text{-}1(7\text{–}36)NH2}}$. Finally, to determine the actual bias of each agonist for different signaling pathways, the $\Delta\log(\tau/K_A)$ values were evaluated statistically between the pathways. The ligand bias of an agonist for one pathway, $j1$, over another, $j2$, is given as

$$Bias = 10^{\Delta\Delta log\left(\frac{\tau}{K_A}\right)j1 - j2} \tag{4}$$

where

$$\Delta\Delta log\left(\frac{\tau}{K_A}\right)_{j1-j2} = log(bias) = \Delta log\left(\frac{\tau}{K_A}\right)_{j1} - \Delta log\left(\frac{\tau}{K_A}\right)_{j2} \tag{5}$$

A lack of functional selectivity will thus result in bias values not substantially different from the value of 1 between pathways and, hence, log (bias) values not significantly different from zero. To account for the propagation of error associated with the determination of composite parameters, the following equation was used.

$$Pooled\ SE = \sqrt{(SE2)^1 + (SE2)^2} \tag{6}$$

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 et al., 2007; Aurelio et al., 2009)

$$E = \frac{E_{\mathrm{m}}(\tau_{\mathrm{A}}[A](K_B + \alpha\beta[B]) + \tau_B[B]\mathrm{EC}_{50})^n}{\mathrm{EC}_{50}^n(K_B + [B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]\mathrm{EC}_{50})^n} \tag{7}$$

$$E = \frac{E_{\mathrm{m}}(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + [B]K_A + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \tag{8}$$

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 measures of the ligands' respective signaling efficacies that incorporate receptor expression levels and efficiency of stimulus-response coupling. Equation 4 was used in interaction studies performed between allosteric ligand (BEPT) and a full agonist (in cAMP and pERK1/2 assays), while eq. 5 was used when the BEPT was interacted with a partial agonist (in iCa^{2+}, β-Arr1, and β-Arr2 assays). This is so because eq. 4 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 $P < 0.05$.

Results

Small Molecules Ligands and Peptides Differentially Couple the GLP-1R to Cellular Effectors. The ability of a GPCR to couple to multiple intracellular signaling components is a requirement for stimulus bias. Like most GPCRs, the GLP-1R couples to different classes of heterotrimeric G proteins, including $G\alpha_s$, $G\alpha_q$, and $G\alpha_i$, as well as various other signaling and regulatory proteins such as the β-Arrs. In this study, the selective GLP-1R small molecules, BETP (Sloop et al., 2010), Compound 2 (Knudsen et al., 2007), TT15 (Rao, 2009), Boc5 (Chen et al., 2007), and a modified GLP-1 analog (BMS21) (Mapelli et al., 2009) (Fig. 1) were assessed for their ability to activate various intracellular signaling pathways. These included cAMP (as a surrogate of canonical $G\alpha_s$ coupling), iCa^{2+} mobilization (as a measure of $G\alpha_q$, and to some extent $G\alpha_i$ coupling), pERK1/2 [as a downstream measure of various convergent pathways (G protein and non-G protein-mediated)], and recruitment of the regulatory proteins β-Arr1 and β-Arr2.

GLP-1(7–36)NH_2 can activate all five of these signaling/regulatory pathways in the Flp-In-CHO GLP-1R cell line selected for this study; however, none of the small molecules or the 11-mer peptide (BMS21) tested were able to fully mimic the actions of the native peptide ligand (Fig. 2; Table 1). BMS21 had a much lower potency than GLP-1(7–36)NH_2; however, this ligand displayed higher efficacy for cAMP signaling with an increased E_{max} (Fig. 2; Table 1). Interestingly, this small peptide displayed a similar potency in pERK1/2 and iCa^{2+}-mobilization assays as in the cAMP assay; however, in these instances the observed E_{max} was dramatically lower than that of GLP-1(7–36)NH_2. In addition, BMS21

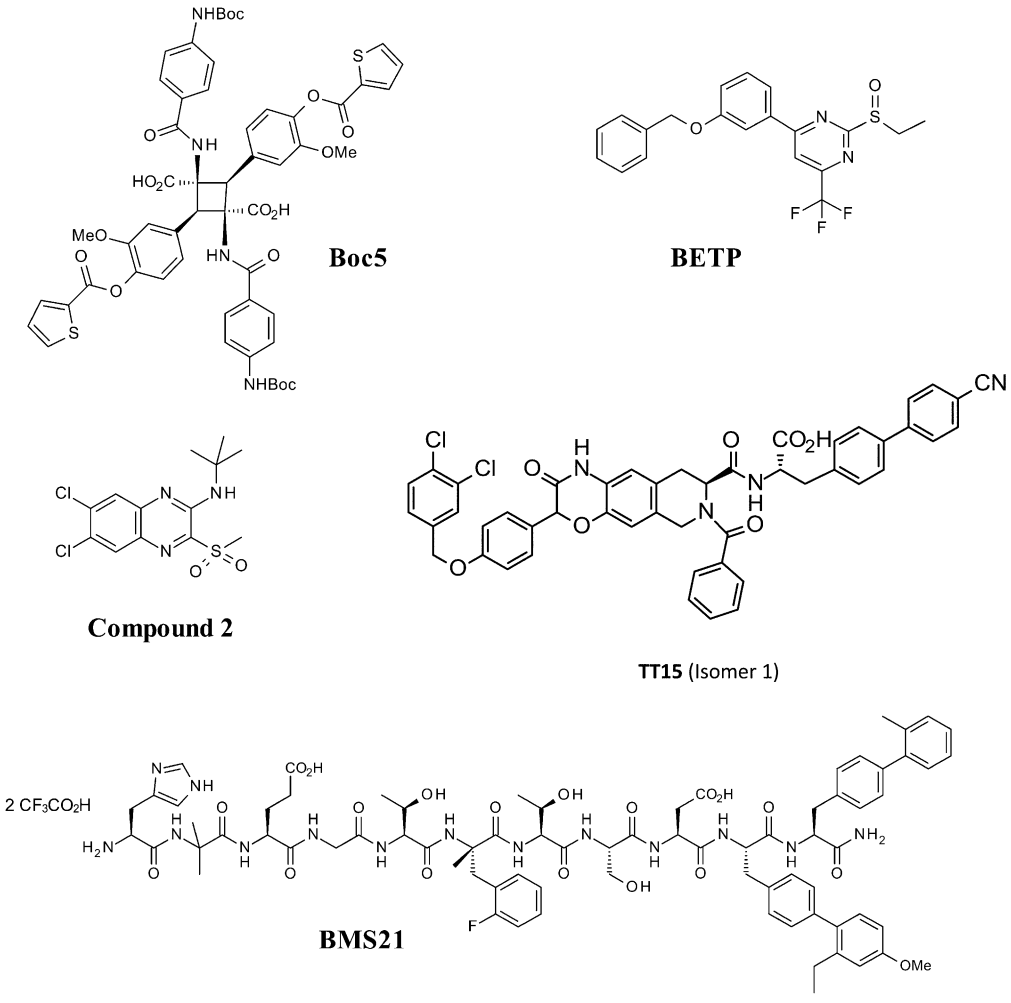

Fig. 1. Small molecule ligand structures. Structures of small molecule ligands used in this study.

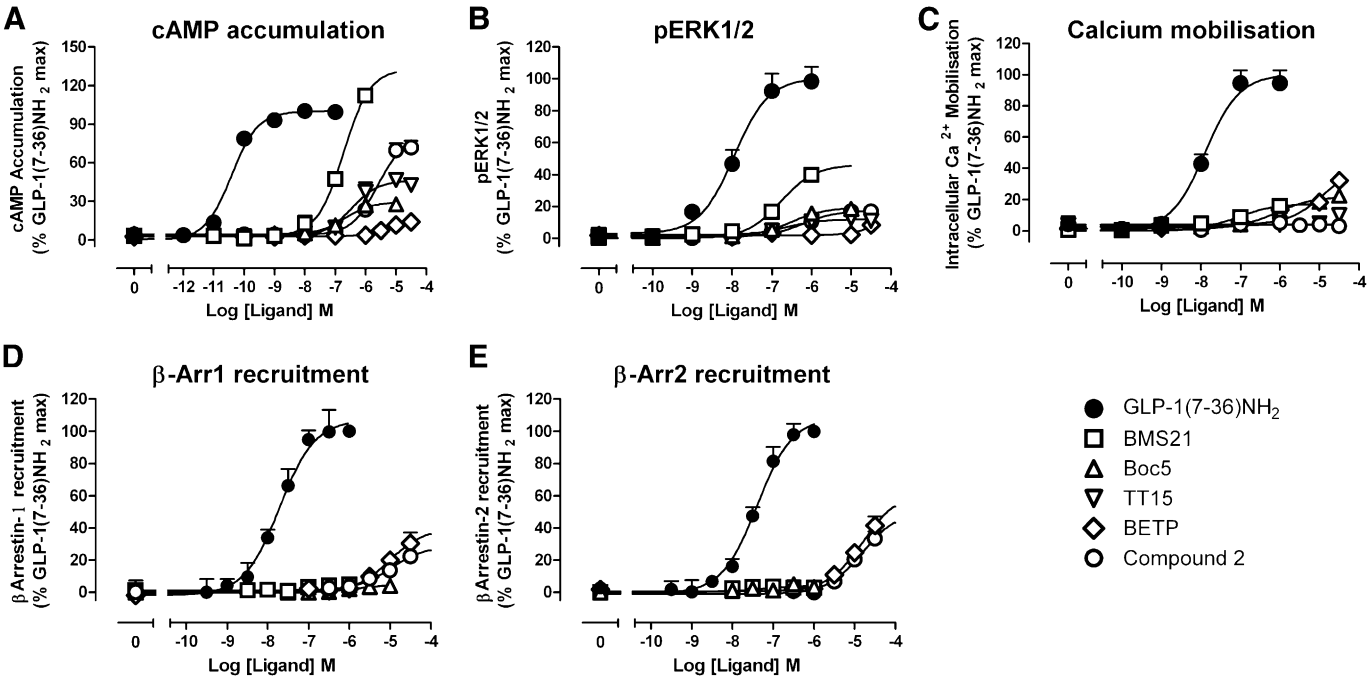

Fig. 2. Signaling profiles of GLP-1R ligands. Dose response curves for cAMP accumulation (A), pERK1/2 (B), iCa^{2+} mobilization (C), β-Arr1 recruitment (D), and β-Arr2 (E) recruitment for GLP-1(7–36)NH_2, BMS21, Boc5, TT15, BETP, and Compound 2. Data are normalized to the response elicited by GLP-1(7–36)NH_2 and analyzed using a three-parameter logistic equation. All values are means ± S.E.M. of three to four independent experiments conducted in duplicate.

was unable to recruit β-Arrs within the tested concentration range (Fig. 2; Table 1) suggesting that although this peptide is similar to the N-terminal portion of the native ligand, this in itself is insufficient to mimic the functions of full length GLP-1(7–36)NH_2.

In agreement with previous studies, the nonpeptidic compound Boc5 was able to increase cAMP with a lower potency and efficacy than GLP-1(7–36)NH_2 and BMS21 (Fig. 2A; Table 1). Boc5 also had similar efficacy in pERK1/2 and iCa^{2+}-mobilization assays. No β-Arr recruitment could be detected for this ligand. TT15 displayed a similar potency but a marginally higher E_{max} for cAMP signaling compared with Boc5; however, it displayed a weaker pERK1/2 response and no iCa^{2+} mobilization was detectable (Fig. 2; Table 1). Unfortunately, this ligand nonspecifically interfered with BRET assay for β-Arr recruitment and therefore characterization of TT15 for β-Arr recruitment could not be performed. Compound 2 and BETP are low potency agonists for cAMP accumulation with BETP displaying weak partial agonism and Compound 2 strong partial agonism. Both compounds

TABLE 1

Differential effects of peptide/small molecule agonists of the human GLP-1R in cAMP accumulation, iCa^{2+} mobilization, pERK1/2, and β-arrestin1 and β-arrestin2 recruitment in Flp-In-CHO cells stably expressing the human GLP-1R

pEC50 values are the negative logarithm of the concentration of agonist that produces half the maximal response. E_{max} represents the maximal response normalized to that of GLP-1(7–36)NH_2. All values are mean ± S.E.M. of three to five independent experiments conducted in duplicate.

Signaling Pathway		Ligand					
		GLP-1(7–36)NH_2	BMS21	Boc5	TT15	BETP	Compound 2
cAMP	pEC_{50}	10.4 ± 0.1	6.7 ± 0.6	6.7 ± 0.2	6.5 ± 0.2	5.2 ± 0.2	5.6 ± 0.1
	E_{max}	100 ± 2	132 ± 6	30 ± 2	46 ± 3	17 ± 2	81 ± 4
pERK1/2	pEC_{50}	7.9 ± 0.1	6.8 ± 0.1	6.5 ± 0.1	6.7 ± 0.3	NR	6.2 ± 0.1
	E_{max}	100 ± 5	46 ± 2	19 ± 1	12 ±	NR	18 ± 1
iCa^{2+}	pEC_{50}	7.9 ± 0.1	7.0 ± 0.3	6.0 ± 0.3	NR	5 ± 0.3	NR
	E_{max}	100 ± 5	17 ± 3	22 ± 2	NR	42 ± 10	NR
β-Arr1	pEC_{50}	7.7 ± 0.1	NR	NR	ND	5.0 ± 0.2	5.0 ± 0.2
	E_{max}	100 ± 6	NR	NR	ND	40 ± 7	30 ± 5
β-Arr2	pEC_{50}	7.4 ± 0.1	NR	NR	ND	5.0 ± 0.3	4.8 ± 0.2
	E_{max}	100 ± 5	NR	NR	ND	63 ± 15	51 ± 0.2

β-Arr, beta arrestin; BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; Boc5, 1,3-bis [[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid; Compound 2, 6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; ERK1/2, extracellular signal-related kinases 1 and 2; iCa^{2+}, intracellular calcium; ND, not detected; NR, no response; TT15, (2*S*)-2-[[(8*S*)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid.

also displayed weak partial agonism in pERK1/2; however, in the case of BETP this was barely detectable within the concentration range assessed. Compound 2 displayed no detectable iCa^{2+} response; however, BETP was an agonist for this pathway with an EC_{50} similar to that observed for its cAMP response, and with an E_{max} of 42 ± 10% of that of GLP-1(7–36)NH_2. However, both ligands were weak agonists for β-Arr1 and β-Arr2 recruitment with E_{max} estimates of 30–40% of the response of GLP-1(7–36)NH_2 (Fig. 2; Table 1).

These effects on ligand bias can be readily observed in bias plots, which display the response observed to equimolar conentrations of ligand for one pathway relative to another (Fig. 3). More importantly, this relative bias can be quantified by calculation of bias factors to compare relative bias to the reference ligand, in this case the primary endogenous ligand GLP-1(7–36)NH_2 (Table 2). It is apparent for all of the small molecule ligands that the GLP-1R shows less preference for coupling to cAMP over other pathways in comparison with activation by GLP-1(7–36)NH_2. However some ligands heavily change the relative bias. The most dramatic changes in bias are observed with activation by BETP, whereby signaling is biased toward iCa^{2+} mobilization and β-Arr1 and β-Arr2 recruitment over cAMP and pERK1/2 compared with the reference agonist (Fig. 3, B–E, H, and J; Table 2). However, little change in the relative bias between iCa^{2+} and arrestin recruitment was observed (Fig. 3F; Table 2). In contrast BMS21 biases the receptor toward pERK1/2 and cAMP over arrestin recruitment and iCa^{2+} mobilization relative to GLP-1(7–36)NH_2 (Fig. 3, C–E; Table 2). In addition, compared with GLP-1(7–36)NH_2, compound 2 biases the receptor conformations toward β-Arr1 and β-Arr2 recruitment relative to iCa^{2+} (where no response was observed) and cAMP (Fig. 3, F and G; Table 2).

BETP and Compound 2 Selectively Modulate the Affinity of Agonists at the GLP-1R. In agreement with our previous study, Compound 2 displayed probe dependence in that it caused a concentration-dependent increase in affinity of oxyntomodulin, but not of GLP-1(7–36)NH_2, exendin-4, or GLP-1(1–36)NH_2. BETP also displayed the same probe dependence with potentiation of oxyntomodulin affinity and no effect on the other three peptides (Supplemental Fig. 2). The other small molecules did not alter the competition binding profile of ^{125}I-exendin(9–39) in the presence of any peptide ligand tested (Supplemental Fig. 1).

BETP and Compound 2 Differentially Alter Peptide-Mediated GLP-1R Signaling Bias. Analysis of the interaction between BETP and orthosteric peptide ligands with the allosteric operational model revealed BETP differentially modulated GLP-1R agonist intrinsic efficacy in a ligand and pathway-dependent manner. (Figs. 4–7; Table 3). Combined affinity-efficacy ($\alpha\beta$) estimates for cAMP were consistent with affinity cooperativity estimates from the binding studies (Fig. 4; Supplemental Fig. 2; Table 3). Thus, exendin-4, GLP-1(7–36)NH_2, and GLP-1(1–36)NH_2 displayed neutral cooperativity for both binding and cAMP accumulation,

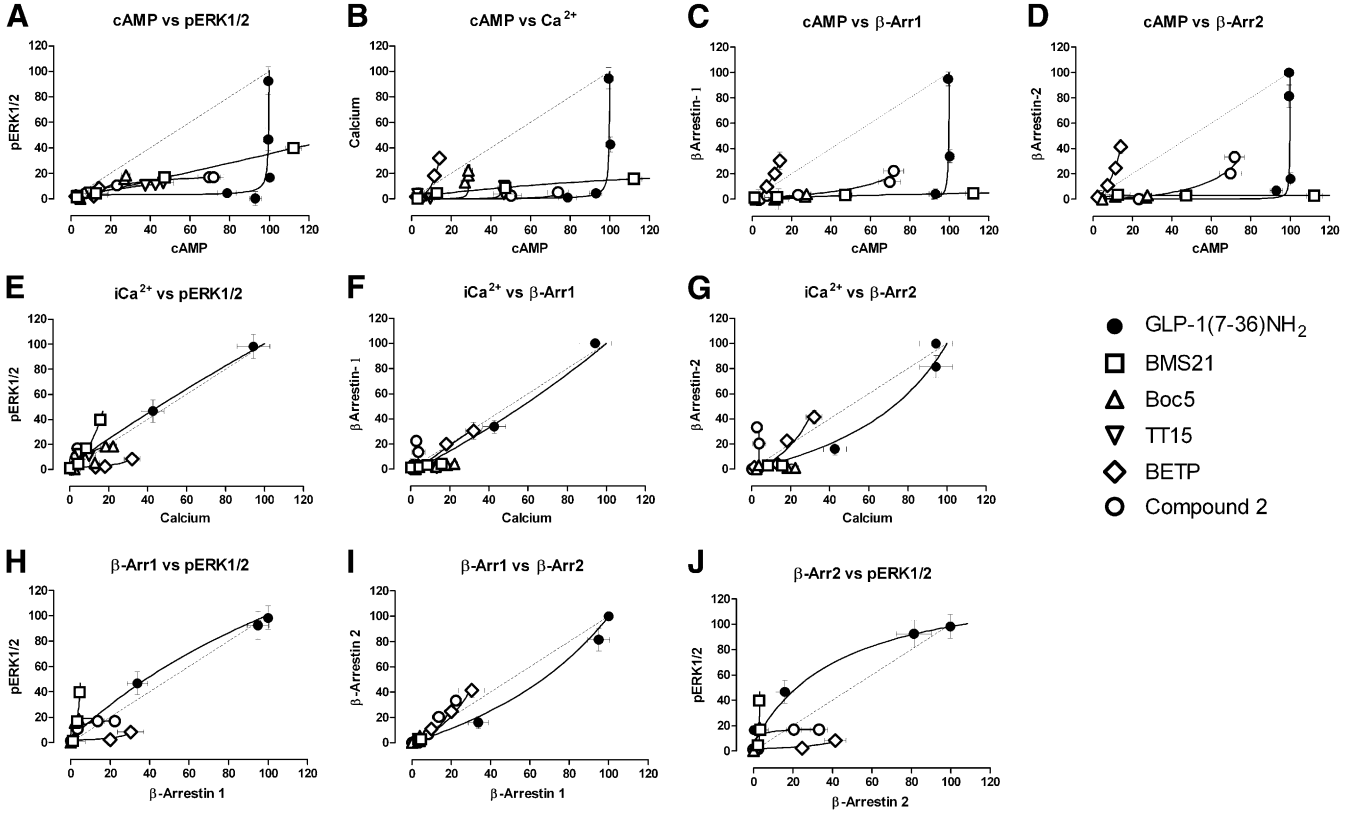

Fig. 3. Synthetic ligands display stimulus bias relative to the endogenous ligand GLP-1(7–36)NH_2. Bias plots of cAMP versus pERK1/2 (A), cAMP versus iCa^{2+} mobilization (B), cAMP versus β-Arr1 (C), cAMP versus β-Arr2 (D), iCa^{2+} versus pERK1/2 (E), iCa^{2+} versus β-Arr1 (F), iCa^{2+} versus β-Arr2 (G), β-Arr1 versus pERK1/2 (H), β-Arr1 versus β-Arr2 (I), and β-Arr2 versus pERK1/2 (J). Data for each ligand in each pathway are normalized to the maximal response elicited by GLP-1(7–36)NH_2, and analyzed with a three-parameter logistic equation with 150 points defining the curve.

TABLE 2

Stimulus bias exhibited by ligands relative to the reference agonist GLP-1(7–36)NH_2

Data were analyzed using an operational model of agonism as defined in eq. 4 to estimate log τ_c/K_A ratios. Changes in log τ_c/K_A ratios were calculated to provide a measure of the degree of stimulus bias exhibited between different signaling pathways relative to that of the reference agonist (GLP-1(7–36)NH_2). Values are expressed as means ± S.E.M. of three to five independent experiments conducted in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

Pathway 1: Pathway 2		Ligand					
		GLP-1(7–36)NH_2	BMS21	Boc5	TT15	BETP	Compound 2
pERK1/2:	cAMP	0 ± 0.11 (1)	0.83 ± 0.34 (6.7)	1.77 ± 0.49* (59)	1.45 ± 0.55 (28)	1.09 ± 0.60 (12)	1.05 ± 0.43 (11)
	iCa^{2+}	0 ± 0.10 (1)	0.28 ± 0.36 (1.9)	-0.22 ± 0.56 (0.6)	ND	–1.23 ± 0.44 (0.06)	ND
	β-Arr1	0 ± 0.09 (1)	ND	ND	ND	–1.39 ± 0.46 (0.04)*	0.22 ± 0.46 (1.7)
	β-Arr2	0 ± 0.11 (1)	ND	ND	ND	–1.97 ± 0.46 (0.01)*	–0.08 ± 0.44 (0.83)
iCa:	cAMP	0 ± 0.14 (1)	0.60 ± 0.37 (3.9)	0.52 ± 0.28 (3.3)	ND	1.74 ± 0.42 (55)*	ND
	pERK1/2	0 ± 0.10 (1)	–0.28 ± 0.36 (0.53)	0.22 ± 0.56 (1.6)	ND	1.23 ± 0.44 (20)	ND
	β-Arr1	0 ± 0.08 (1)	ND	ND	ND	−0.16 ± 0.16 (0.70)	ND
	β-Arr2	0 ± 0.14 (1)	ND	ND	ND	−1.22 ± 0.16 (0.06)*	ND
β-Arr1:	cAMP	0 ± 0.09 (1)	ND	ND	ND	2.38 ± 0.43 (239)*	1.73 ± 0.22 (54)*
	pERK1/2	0 ± 0.09 (1)	ND	ND	ND	1.39 ± 0.46 (24)*	−0.22 ± 0.46 (0.61)
	iCa^{2+}	0 ± 0.08 (1)	ND	ND	ND	0.16 ± 0.16 (1.43)	ND
	β-Arr2	0 ± 0.10 (1)	ND	ND	ND	−0.58 ± 0.19 (0.26)	−0.3 ± 0.24 (0.50)
β-Arr2:	cAMP	0 ± 0.11 (1)	ND	ND	ND	2.96 ± 0.43 (918)*	2.03 ± 0.17 (108)*
	pERK1/2	0 ± 0.11 (1)	ND	ND	ND	1.97 ± 0.46 (93)*	0.08 ± 0.44 (1.2)
	iCa^{2+}	0 ± 0.16 (1)	ND	ND	ND	1.22 ± 0.16 (17)*	ND
	β-Arr1	0 ± 0.10 (1)	ND	ND	ND	0.58 ± 0.19 (3.8)	0.3 ± 0.24 (2.0)

β-Arr, beta arrestin; BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; Boc5, 1,3-bis [[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis [3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid; Compound 2, 6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; ERK1/2, extracellular signal-related kinases 1 and 2; iCa^{2+}, intracellular calcium; ND, not defined; TT15, (2*S*)-2-[[(8*S*)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid.

* $P < 0.05$.

whereas BETP potentiated oxyntomodulin affinity and cAMP responses (Fig. 4; Supplemental Fig. 2). In contrast, BETP showed significant negative cooperativity with exendin-4, GLP-1(7–36)NH_2, and GLP-1(1–36)NH_2 for coupling to pERK1/2 and neutral/weak negative cooperativity with oxyntomodulin for this pathway. In iCa^{2+}-mobilization assays, BETP displayed positive cooperativity with exendin-4 and to a lesser extent GLP-1(7–36)NH_2; however, neutral cooperativity with oxyntomodulin was observed (Fig. 6). Assessment of β-Arr recruitment revealed neutral cooperativity between BETP and exendin-4 for both β-Arr1 and β-Arr2 and neutral cooperativity for GLP-1(7–36)NH_2 in recruiting β-Arr1 (Fig. 7; Table 3). However, weak potentiation of β-Arr2 and of both β-Arr1 and β-Arr2 recruitment was observed for GLP-1(7–36)NH_2 and oxyntomodulin, respectively, in the presence of BETP (Fig. 7; Table 3). These data indicate that BETP can engender stimulus bias at the level of the signaling pathway in a ligand-dependent manner.

Functional interaction assays for cAMP accumulation and iCa^{2+} mobilization between each peptide ligand and Compound 2 confirmed previous findings (Koole et al., 2010); Compound 2 potentiated oxyntomodulin-induced cAMP responses but not intracellular calcium mobilization (Supplemental Figs. 3 and 4). In contrast, neutral cooperativity was observed between Compound 2 and the other three peptides in both pathways. Interaction assays for the pERK1/2 experiments included higher concentrations of Compound 2 than previously published, which revealed significant negative cooperativity of Compound 2 on exendin-4-mediated pERK1/2 responses (Supplemental Fig. 5). A similar trend was observed for both the full-length and truncated GLP-1 peptides (and to a lesser extent oxyntomodulin), although this negative cooperativity did not reach statistical significance. In contrast, Compound 2 displayed positive cooperativity with exendin-4, GLP-1(7–36)NH_2, and oxyntomodulin for recruitment of both β-Arr1 and β-Arr2. The estimated cooperativity factors ($\alpha\beta$) revealed that this potentiation was greater for GLP-1(7–36)NH_2 and oxyntomodulin than that of exendin-4 (Fig. 8). Like BETP, Compound 2 can also generate stimulus bias in a probe-dependent manner; however, it is important to note that these two allosteric ligands engender significantly different signaling profiles that only manifest when multiple signaling pathways are explored.

GLP-1(1–36)NH_2 did not display agonism in either iCa^{2+}-mobilization assays or in recruitment of β-Arrs either in the presence or absence of either BETP or Compound 2.

In contrast to BETP and Compound 2, the small molecules TT15, Boc5, and the BMS21 peptide did not modulate any signaling pathway mediated by any of the GLP-1 peptide agonists (Supplemental Figs. 6–8). These compounds at high concentrations (particularly evident with BMS21) have characteristics consistent with a competitive mode of action with GLP-1- and GLP-1-related peptide agonists, which suggests these small ligands may share at least a partially overlapping binding site with the orthosteric pocket.

BETP and Compound 2 Can Potentiate Responses to BMS21, TT15, and Boc5. Consistent with the evidence above indicating at least a partial overlap in binding interactions formed by TT15 and BMS21 with orthosteric ligands, these two ligands when tested for interaction with each other in a cAMP assay displayed behavior consistent with a competitive interaction (Supplemental Fig. 9). In addition, BETP and Compound 2 strongly potentiated cAMP responses mediated by both TT15 and the small peptide, BMS21 (Fig. 9; Table 4). Interestingly, BETP also potentiated Boc5-mediated cAMP responses (Fig. 9; Table 4); however, only weak modulation was observed using Compound 2 (Fig. 9; Table 4). This is particularly interesting as Boc5, when interacted in a cAMP assay with either TT15 or BMS, had a profile consistent with competitive behavior between the two ligands (Supplemental Fig. 9). This indicates that although both ligands may bind in a site partially overlapping

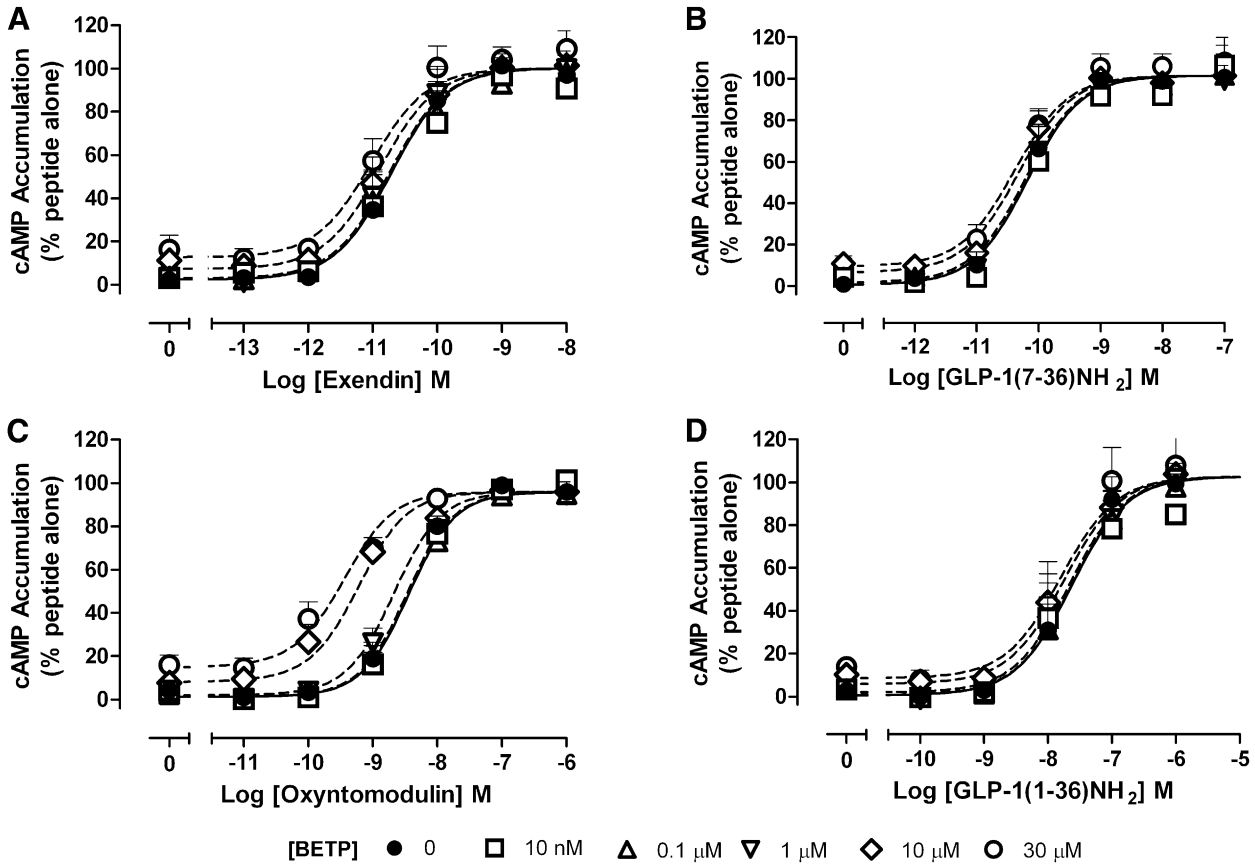

Fig. 4. BETP displays positive allosteric effects on GLP-1R-mediated cAMP accumulation in an agonist-dependent manner. Concentration response curves were generated for exendin-4 (A), GLP-1(7–36)NH_2 (B), oxyntomodulin (C), or GLP-1(1-36)NH_2 (D) in the absence and presence of increasing concentrations of BETP in Flp-In-CHO cells stably expressing the human GLP-1R. The curves represent the best global fit of an operational model of allosterism (eq. 4). All values are mean ± S.E.M. of three to four independent experiments performed in duplicate. Panel C reproduced from Willard et al. (2012b).

the orthosteric site, the cooperativity between the site of Compound 2 binding and Boc5 is different from that of TT15 and BMS21. In addition, the differential degrees of cooperativity induced by the two structurally distinct modulators, BETP and Compound 2, indicate that these two compounds interact differentially with the GLP-1R.

Discussion

The GLP-1R is a major therapeutic target for the treatment of type 2 diabetes, however, despite the success of natural or modified GLP-1R-binding peptides for clinical treatment, low molecular weight, orally active compounds are still pursued as the preferred therapeutic approach. Traditionally, these types of molecules were designed to mimic the properties of the natural ligand by targeting the orthosteric site and this approach has been successful for many GPCR targets (Black, 1989). However, there are many cases where this has been unsuccessful, in particular for non-family A GPCRs.

Orthosteric peptide ligands for family B GPCRs bind predominantly to the large N-terminal domain prior to initiating receptor activation (Hoare, 2005). This is mechanistically different from many family A GPCRs, whose ligands primarily make contact within the transmembrane domain. Due to the size of peptide ligands and their mechanism of receptor activation, the discovery of surrogate small molecule agonists that mimic these actions has been difficult. However, several groups have recently reported small molecule nonpeptide and smaller peptide fragments that act as GLP-1R agonists or positive allosteric modulators. In this study we have revealed significant signaling bias induced by these compounds when compared with the predominant endogenous peptide, indicating that small ligands may not be able to fully mimic the actions of larger peptide hormones. In addition, we show that allosteric modulation is complex, with pathway-dependent modulation of receptor response that is determined by the combination of orthosteric ligand and allosteric ligand used. This emphasizes the need for broad elucidation of mechanism of action when developing allosteric compounds.

Activation by peptide ligands predominantly couples the GLP-1R to $G\alpha_S$-proteins, leading to an increase in cAMP. This is the best studied pathway of the GLP-1R and is crucial for enhancing glucose-dependent insulin secretion (Baggio and Drucker, 2007). However, like many GPCRs, the GLP-1R elicits signals via diverse pathways, including iCa^{2+} mobilization and pERK1/2, in addition to coupling to regulatory proteins such as β-Arrs that can activate other effectors (Montrose-Rafizadeh et al., 1999; Sonoda et al., 2008). Each of these pathways has been linked to physiologic effects of GLP-1. iCa^{2+} mobilization can significantly modulate the magnitude of insulin secretion, and β-Arr1 also has a role in insulin

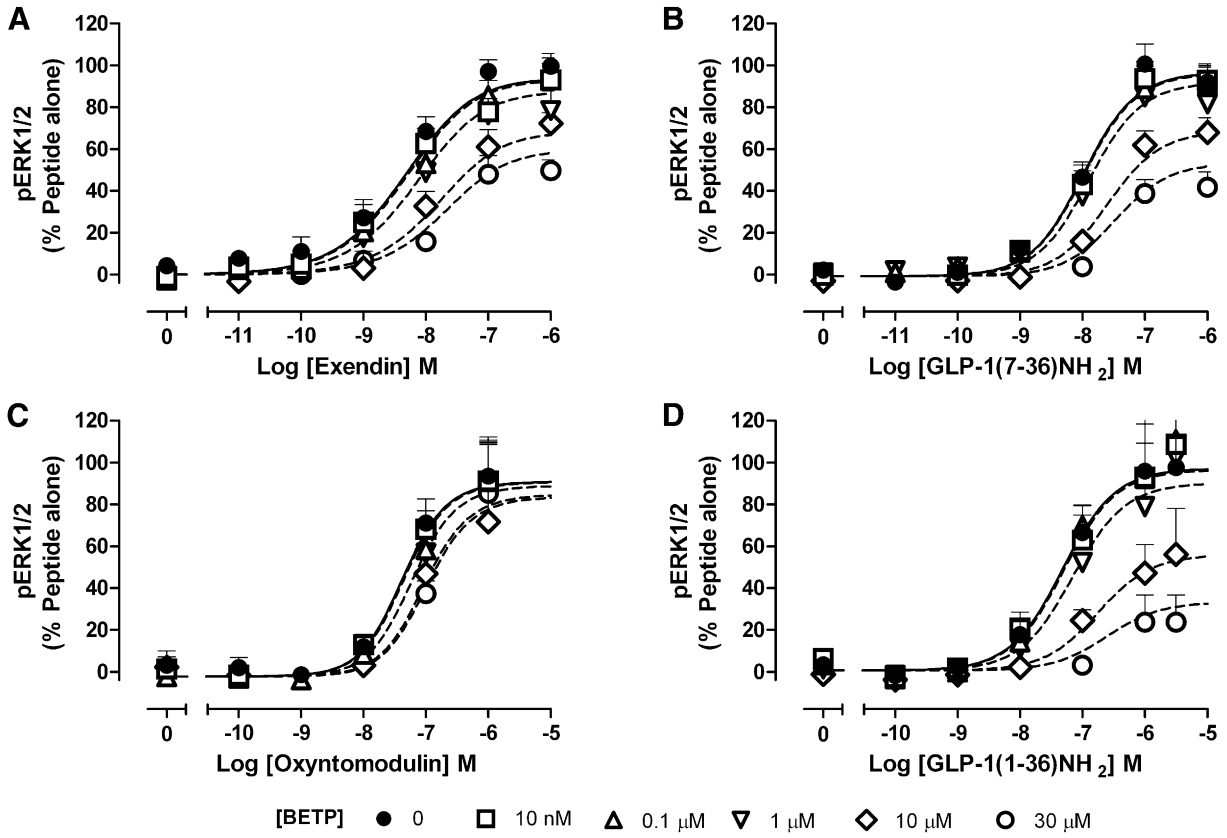

Fig. 5. BETP displays negative allosteric effects on GLP-1R-mediated pERK1/2 by peptide ligands. Concentration response curves were generated for exendin-4 (A), GLP-1(7–36)NH_2 (B), oxyntomodulin (C) or GLP-1(1–36)NH_2 (D) in the absence and presence of increasing concentrations of BETP in Flp-In-CHO cells stably expressing the human GLP-1R. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of three to four independent experiments performed in duplicate. Panel C reproduced from Willard et al. (2012b).

secretion, although the molecular mechanism of this regulation is poorly understood. Sustained effects on gene transcription and the preservation of β-cell mass involve multiple signaling pathways; both cAMP-dependent and -independent; the latter include activation of mitogen-activated kinases, such as ERK1/2. It is clear that the physiological response downstream of GLP-1R activation is a composite of the interplay of various signaling pathways, but even for those that have been identified, the extent and magnitude to which these effectors contribute to the physiological signaling profile and the ideal combination of these that lead to a therapeutically beneficial output has yet to be established.

Evaluation of signaling across five pathways (cAMP, pERK1/2, iCa^{2+} mobilization, β-Arr1, and β-Arr2 recruitment) demonstrated that, in comparison with the reference ligand [GLP-1(7–36)NH_2], all of the small ligands, with the exception of BETP, coupled most strongly to cAMP production. In addition, for BMS21, TT15, and Boc5, the relative order of efficacy for the five pathways was similar to GLP-1(7–36)NH_2 (Fig. 1; Table 1). Despite this, each of the

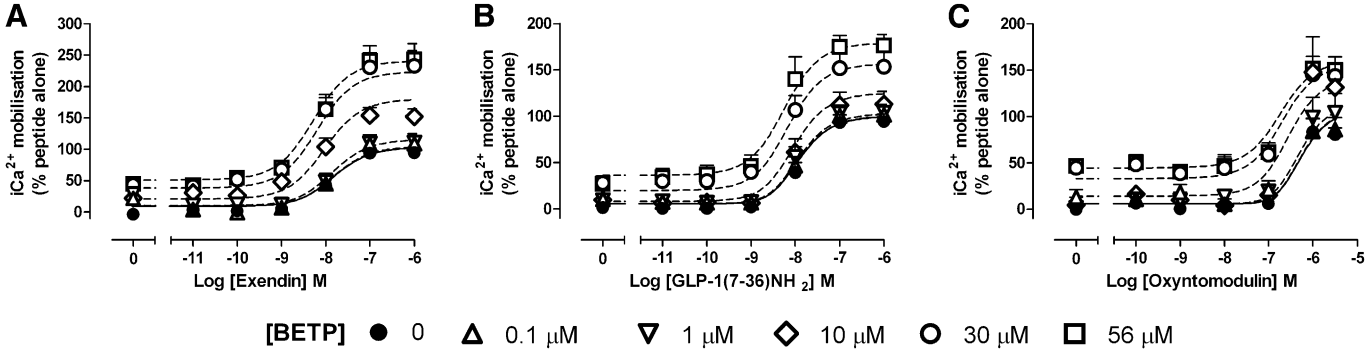

Fig. 6. BETP positively modulates GLP-1R-mediated iCa^{2+} mobilization by peptide ligands. Concentration response curves were generated for exendin-4 (A), GLP-1(7–36)NH_2 (B) or oxyntomodulin (C) in the absence and presence of increasing concentrations of BETP in Flp-In-CHO cells stably expressing the human GLP-1R. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of three to four independent experiments performed in duplicate. Panel C reproduced from Willard et al. (2012b).

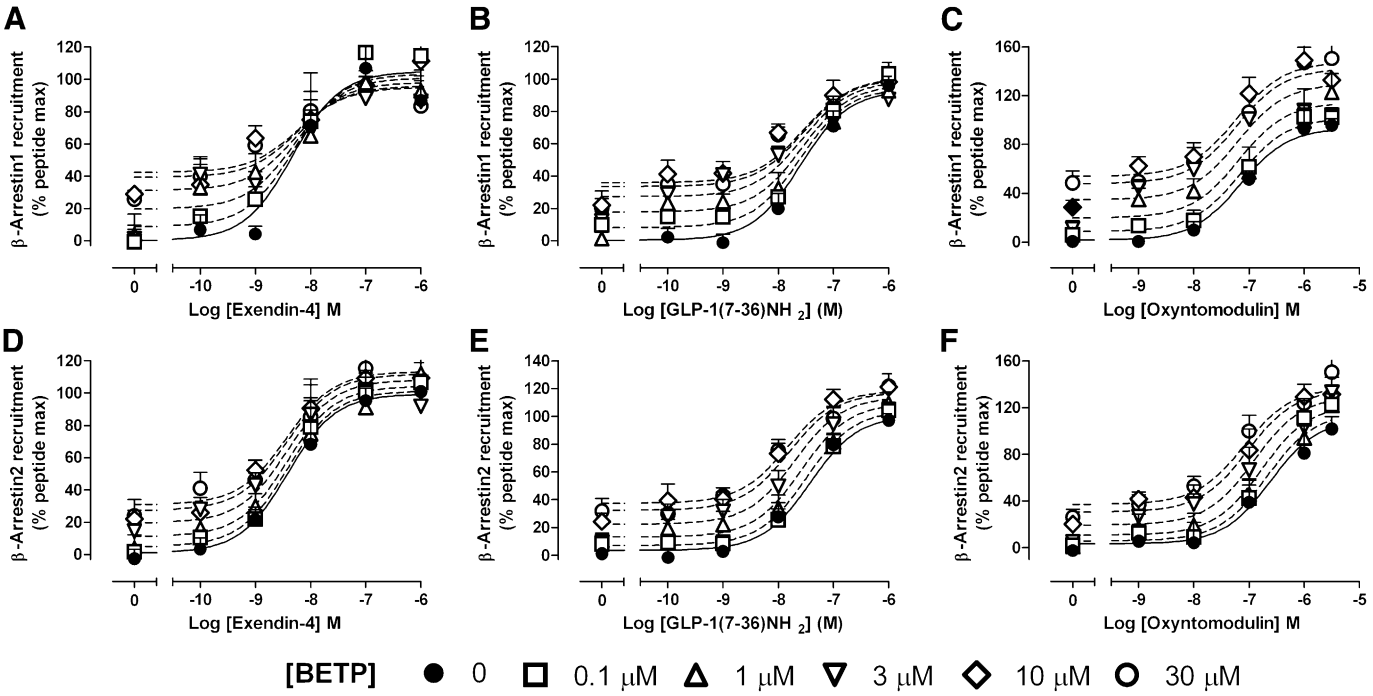

Fig. 7. BETP does not significantly alter GLP-1R-mediated recruitment of β-arrestins by peptide ligands. Concentration response curves were generated for exendin-4 (A and D), GLP-1(7–36)NH_2 (B and E), or oxyntomodulin (C and F) in the absence and presence of increasing concentrations of BETP for β-Arr1 (A–C) and β-Arr2 (D–F) recruitment. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of four to five independent experiments performed in duplicate. Panel C reproduced from Willard et al. (2012b).

ligands showed elements of signal bias, with all three having less preference for cAMP relative to pERK1/2, but no significant change when comparing the preference between all other pathways (Table 2). However, Compound 2 displayed significant signal bias with less preference for cAMP signaling relative to iCa^{2+} mobilization, β-Arr1, or β-Arr2. Interestingly BETP displayed a very different profile to GLP-1(7–36)NH_2, as this compound heavily biased GLP-1R signaling to β-Arr1, β-Arr2, and iCa^{2+} mobilization relative to cAMP and pERK1/2. The response was also biased toward β-Arr1 recruitment and iCa^{2+} mobilization over β-Arr2 (Table 2). The ability of individual ligands to differentially activate the GLP-1R to produce distinct functional profiles may provide a unique opportunity in drug development, with the potential to sculpt receptor signaling to target physiologically important responses and exclude those that do not provide beneficial outputs.

This concept also extends to allosteric modulation of orthosteric ligand responses. In addition to small molecules displaying differential intrinsic efficacy profiles, if they bind allosterically, they can also differentially modulate peptide (both endogenous and exogenous) responses in a pathway-specific manner. Therefore, determining the modulatory profile of small molecule ligands in numerous functional outputs and using multiple orthosteric ligands is important, especially when the endogenous systems involve the interplay

TABLE 3

Functional cooperativity estimates for the interaction between BETP or Compound 2 and GLP-1R peptide ligands

Data derived from analysis of interaction concentration-response curves with an operational model of allosterism as defined in eqs. 4 and 5. pKb values are the negative logarithms for the functional affinity of the allosteric ligands; log αβ represents the composite cooperativity between the allosteric ligand and the orthosteric peptide ligand. Antilogarithms are shown in parentheses. Values represent the mean ± S.E.M. of four to six independent experiments performed in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

Pathway	Allosteric Ligand	pKb	Log αβ (αβ)			
			Exendin-4	GLP-1(7–36)NH_2	Oxyntomodulin	GLP-1(1–36)NH_2
cAMP	BETP	5.01 ± 0.04	0.45 ± 0.20 (2.8)	0.31 ± 0.18 (2.0)	1.21 ± 0.17 (16)*	0.20 ± 0.12 (1.6)
	Cpd2	5.43 ± 0.29	0.24 ± 0.30 (1.7)	0.22 ± 0.28 (1.7)	1.48 ± 0.27 (29)*	0.31 ± 0.17 (2.0)
pERK1/2	BETP	5.46 ± 0.29	–0.90 ± 0.21 (0.13)*	–1.03 ± 0.23 (0.09)*	−0.44 ± 0.19 (0.36)	−1.85 ± 0.88 (0.01)
	Cpd2	5.29 ± 0.19	–0.77 ± 0.21 (5.9)	–0.48 ± 0.17 (0.33)	−0.21 ± 0.13 (0.62)	−0.44 ± 0.20 (0.36)
iCa^{2+}	BETP	4.83 ± 0.16	1.0 ± 0.26 (10)*	0.58 ± 0.19 (3.8)	0.23 ± 0.11 (1.7)	NR
	Cpd2	5.58 ± 0.38	0.28 ± 0.15 (1.9)	–0.20 ± 0.15 (0.63)	0.14 ± 0.16 (1.4)	NR
β-Arr1	BETP	5.42 ± 0.17	–0.05 ± 0.04 (0.89)	–0.01 ± 0.02 (1.0)	0.40 ± 0.17 (2.5)	NR
	Cpd2	5.27 ± 0.18	0.72 ± 0.18 (5.2)*	1.07 ± 0.19 (12)*	1.05 ± 0.14 (11)*	NR
β-Arr2	BETP	5.38 ± 0.16	0.18 ± 0.26 (1.5)	0.67 ± 0.18 (4.7)	0.54 ± 0.19 (3.5)	NR
	Cpd2	5.30 ± 0.19	0.69 ± 0.20 (4.9)*	1.06 ± 0.13 (11)*	0.99 ± 0.19 (10)*	NR

β-Arr, beta arrestin; BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; Compound 2, 6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; iCa^{2+}, intracellular calcium; NR, no response; pKb, negative logarithms for the functional affinity of the allosteric ligands.
* $P < 0.05$.

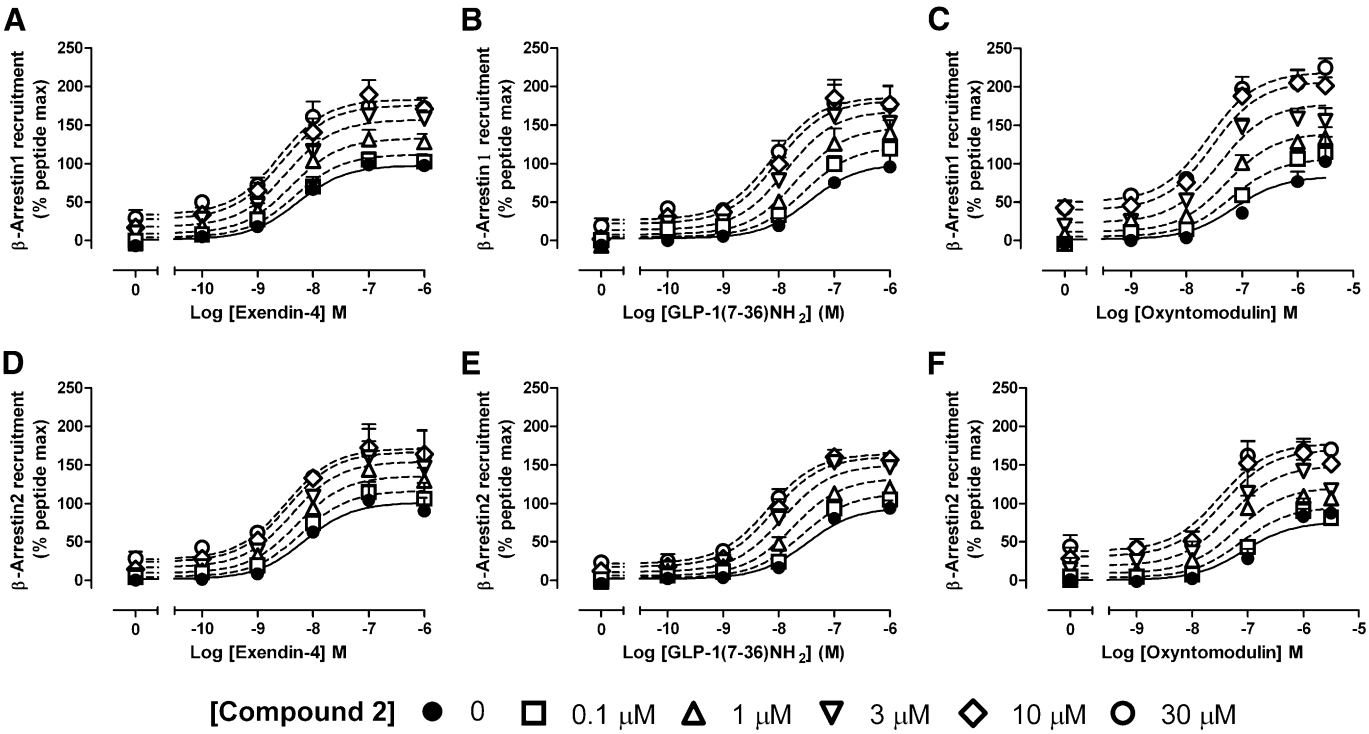

Fig. 8. Compound 2 potentiates GLP-1R-mediated recruitment of β-arrestins by peptide ligands. Concentration response curves were generated for exendin-4 (A and D), GLP-1(7–36)NH_2 (B and E) or oxyntomodulin (C and F) in the absence and presence of increasing concentrations of Compound 2 for β-Arr1 (A–C) and β-Arr2 (D–F) recruitment. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of four to five independent experiments performed in duplicate.

of many natural ligands and several signaling pathways to elicit physiological consequences. Compound 2 engendered significant bias in the response mediated by oxyntomodulin with selective enhancement of cAMP, β-Arr1, and βArr2; however, for GLP-1(7–36)NH_2, only β-Arr responses were enhanced. BETP also engendered significant stimulus bias in a probe-dependent manner, with selective enhancement of oxyntomodulin-mediated cAMP responses and to a smaller extent β-Arr-1 and-2, but only iCa^{2+} mobilization and β-Arr2 responses were weakly enhanced when GLP-1(7–36)NH_2 was cobound, while a strong negative effect on pERK1/2 was observed. When considering the clinically used exendin-4, the bias was again different; in this case only iCa^{2+} mobilization was significantly enhanced, with negative cooperativity seen for pERK1/2. This revealed that GLP-1R conformations induced by the cobinding of an allosteric modulator and orthosteric ligand can vastly alter the combined signaling profile of the receptor such that no two combinations of allosteric-orthosteric ligand pair were able to produce the same profile of behavior. From these studies, it is unclear whether Compound 2 and BETP share a common binding pocket, and further elucidation to identify their binding site(s) will be required. However, even if they do occupy the same pocket, the specific interactions formed between these compounds and the receptor is clearly different as they induce very distinct bias in their efficacy and modulatory properties.

This type of behavior, where ligands can alter one pathway while having very different effect on another pathway and differential probe-dependent effects at both acute and regulatory signaling pathways, may provide a therapeutic advantage by allowing fine-tuning of receptor response. However, this also presents a significant challenge, as currently it is not clear what will be the key pathway/combination of pathways that need to be manipulated to provide an ideal therapeutic response. Understanding the activity profiles of small ligands may be key for drug discovery programs. These types of compounds, which display differential efficacy and modulatory profiles, provide us with tools that could potentially be used in an in vivo/ex vivo setting to explore the physiological consequences of biased signaling. Further research is required to fully understand these concepts and ascertain the preferred signaling profile for new and better therapeutics.

The final part of this study identified that Compound 2 and BETP were able to strongly modulate cAMP responses of BMS21 and TT15 at the GLP-1R. Boc5 could also be potentiated but to a lesser extent. Data from our interaction assays also suggest that these compounds behave in a competitive manner with peptide ligands and each other. BMS21 was designed to mimic the N-terminal region of GLP-1, which is proposed to bind to the top of the transmembrane bundle and extracellular loop regions of the receptor. It is also possible that TT15 may bind in a similar region. Boc5 has also been proposed to bind in the extracellular regions of the receptor; however, its binding site may be distinct from that of BMS and TT15 as weaker cooperativity was observed with BETP and Compound 2. These observations could also represent an opportunity to aid in drug optimization. For example, ligands like BMS21, TT15, and Boc5 are less biased agonists than Compound 2 and BETP, and if mimicking the actions of GLP-1(7–36)NH_2 rather than altering the bias of the natural hormone was identified as the best therapeutic

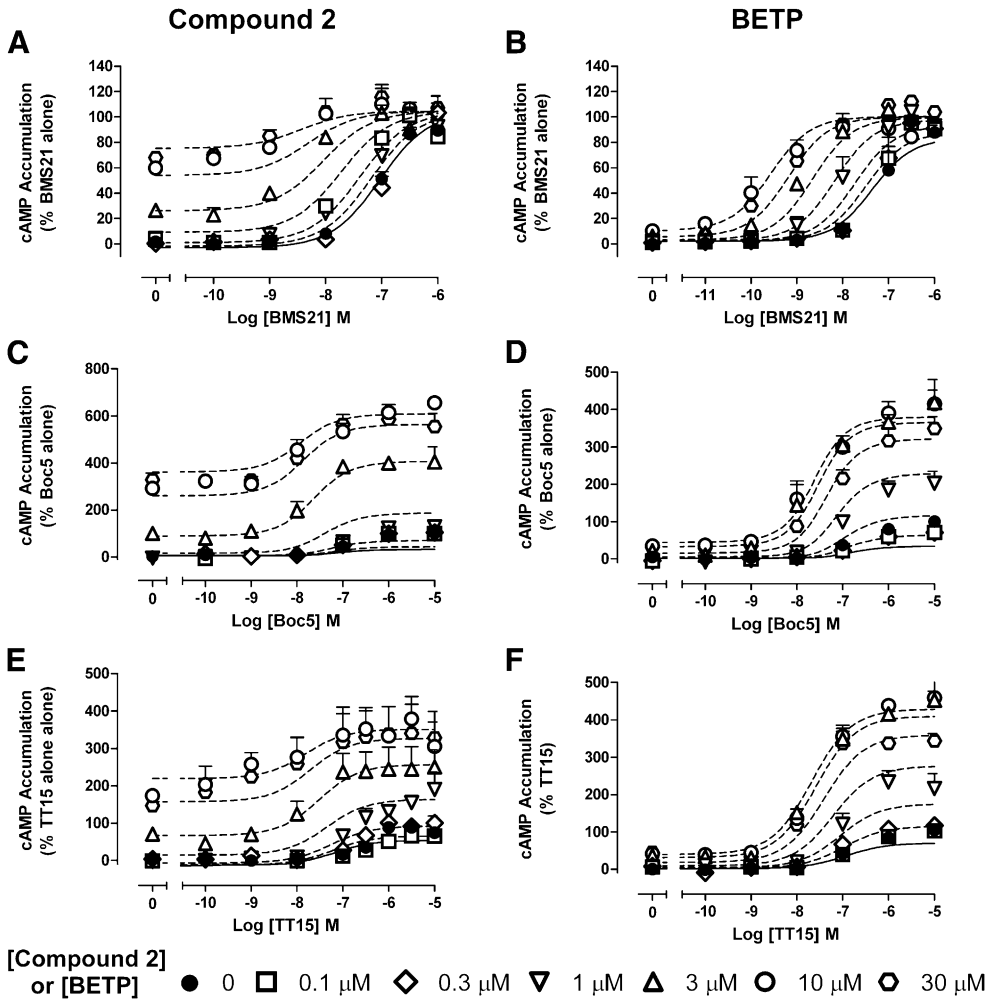

Fig. 9. Compound 2 and BETP potentiate GLP-1R-mediated cAMP accumulation by BMS21, Boc5 and TT15. Concentration response curves were generated for BMS21 (A and B), Boc5 (C and D), or TT15 (E and F) in the absence and presence of increasing concentrations of either Compound 2 (A, C, and E) or BETP (B, D, and F) in Flp-In-CHO cells stably expressing the human GLP-1R. The curves represent the best global fit of an operational model of allosterism (eq. 5). All values are mean ± S.E.M. of three independent experiments performed in duplicate.

approach, then elucidation of the binding sites for these ligands could aid in development of higher affinity drug-like molecules that bind to the same binding pocket. Alternatively, all small ligands identified to date display weak affinity for the GLP-1R that could arguably be due to the limited number of contacts they can form with the receptor (compared with peptide ligands). The ability of one small molecule to enhance the signaling induced by another (and vice versa) may indicate some therapeutic potential for small molecule therapies to be used in combination.

In conclusion, we have demonstrated that small molecule ligands induce biased signaling at the GLP-1R and also bias in the signaling profile of orthosteric ligands. Further work is required to delineate the extent to which such bias exists in a native cellular environment and the in vivo consequences. In recent years, the pace of identifying small molecule GLP-1R ligands has increased and this should aid in the types of studies that may lead to the discovery and development of compounds with the potential to sculpt therapeutics that show greater selectivity and improved therapeutic outcomes.

TABLE 4

Functional cooperativity estimates for the interaction between BETP or Compound 2 and Boc5, TT15, or BMS21

Data derived from analysis of interaction dose-response curves with an operational model of allosterism as defined in eq. 4. Log $\alpha\beta$ represents the composite cooperativity between the allosteric ligand and the orthosteric peptide ligand. Antilogarithms are shown in parentheses. Values represent the mean ± S.E.M. of four to six independent experiments performed in duplicate. Data were analyzed with one-way analysis of variance and Dunnett's post test.

	Log $\alpha\beta$ ($\alpha\beta$)	
	Compound 2	BETP
Boc5	0.84 ± 0.39 (6.9)*	1.28 ± 0.44 (19)*
TT15	1.89 ± 0.41 (78)*	1.66 ± 0.28 (46)*
BMS21	2.09 ± 0.35 (123)*	2.75 ± 0.22 (562)*

BETP, 4-(3-benzyloxyphenyl)-2-ethylsulfinyl-6-(trifluoromethyl)pyrimidine; Boc5, 1,3-bis [[4-(tert-butoxy-carbonylamino)benzoyl]amino]-2,4-bis[3-methoxy-4-(thiophene-2-carbonyloxy)-phenyl]cyclobutane-1,3-dicarboxylic acid; Compound 2, 6.7-dichloro-2-methylsulfonyl-3-tert-butylaminoquinoxaline; TT15, (2*S*)-2-[[(8*S*)-7-benzoyl-3-[4-[(3,4-dichlorophenyl)methoxy]phenyl]-2-oxo-1,6,8,9-tetrahydropyrido[4,3-g][1,4]benzoxazine-8-carbonyl]amino]-3-[4-(4-cyanophenyl)phenyl]propanoic acid.

* $P < 0.05$.

Acknowledgments

The authors thank Dan Kohlman, Mark Tebbe, Mike Coghlan, and Jorge Alsina-Fernandez for technical assistance and support.

Authorship Contributions

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

Conducted experiments: Wootten, Savage.

Contributed new reagents or analytic tools: Willard, Bueno, Sloop.

Performed data analysis: Wootten, Savage.

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

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Address correspondence to: Dr. Patrick M. Sexton, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. [redacted]