To all those who supported me and made my time as a PhD student an enjoyable and memorable experience that I will always cherish

Abstract

G protein-coupled receptors (GPCRs) play fundamental roles in our homeostatic balance through their involvement in numerous physiological processes. In order to maintain their responsiveness to the extracellular environment, a complex process of receptor desensitization and resensitization has evolved. To allow receptors to be resensitized, they must first undergo endocytosis, a process involving numerous adaptor proteins, which facilitate the internalization of the receptor into the cell. The most common method of GPCR internalization engages the clathrin-dependent pathway, where the two most prominent adaptors are βarrestin and AP-2. While there is a relatively clear picture of how these proteins mediate receptor endocytosis, regulatory mechanisms through which these endocytic adaptors may affect or be affected by signalling events is significantly less well described.

The angiotensin II type I receptor is used as the model receptor in our studies, as previous work in our lab characterized some of the protein factors necessary for the endocytic process. While the previous work demonstrated phosphorylation-dependent regulation of βarrestin and AP-2 complex at a putative tyrosine residue, these results were solely *in vitro*, and had not been confirmed in living cells.

As such we generated a polyclonal antibody against this putative phosphorylation site and revealed that not only did this phosphorylation event occur in multiple cell types in response to angiotensin II (AngII) treatment but that the activation of multiple receptors including a non-GPCR, the epidermal growth factor receptor, induced phosphorylation on the β-subunit of AP-2, the main subunit involved in βarrestin binding. My work, which is published in a previous manuscript, also revealed that prevention of this phosphorylation event stabilizes βarrestin/AP-2 complex dramatically. While our initial studies revealed that this phosphorylation event had little impact on receptor endocytosis, new tools have since been developed in the lab. Mainly, we generated a single chain

antibody that can be expressed intracellularly targeting the phosphosite, and demonstrated that binding to this phosphorylated residue results in a prolonged endocytic block. We further established this phosphorylated tyrosine residue as a putative binding motif for certain Src homology 2 (SH2) domain containing proteins as three are capable of binding phosphorylated $\beta_2$adaptin.

Finally, due to the βarrestin-dependent nature of this phosphorylation event, we characterized four AngII analogs with single amino acid substitutions in their octapeptide sequence. Our findings established a correlative relationship between the strength of βarrestin to receptor avidity and the level of extracellular signal-regulated kinase (ERK) activation. Presumably, the differences in avidity would alter the trafficking of the receptor affecting its fate towards recycling or degradation. Furthermore, we establish that the conformation of βarrestin can alter the pathways activated downstream of the receptor resulting in alternative cellular outcomes like cell growth or migration. These results highlight how important regulation of these endocytic adaptors is to overall GPCR fate, not only due to their role in internalization but also for their own signalling potential.

Résumé

Les récepteurs couplés aux protéines G (RCPGs) jouent un rôle fondamental dans notre équilibre homéostatique par leur implication dans de nombreux processus physiologiques. Afin de maintenir leur réactivité à l'environnement extracellulaire, un processus complexe de désensibilisation et resensibilisation des récepteurs a évolué. Afin de permettre aux récepteurs d'être resensibilisés, ils doivent d'abord être internalisés par endocytose, un processus impliquant de nombreuses protéines adaptatrices. La méthode la plus commune de l'internalisation des RCPGs est la voie dépendante à la clathrine, où les deux adaptateurs les plus importants sont βarrestine et AP-2. Bien que nous ayons une image relativement claire de la façon dont ces protéines conduisent à l'endocytose du récepteur, les mécanismes de régulation où ces adaptateurs de l'endocytose peuvent affecter ou être affecté par des processus de signalisation sont nettement moins bien décrits.

Le récepteur de l'angiotensine II de type I est utilisé comme récepteur modèle dans nos études. Les études antérieures de notre laboratoire ont caractérisé certains des facteurs protéiques nécessaires pour le processus d'endocytose. Bien que les études précédentes ont démontré une régulation dépendante à la phosphorylation des complexes βarrestine et AP-2, ces résultats ont été exclusivement in vitro et n'ont pas été confirmés dans les cellules vivantes.

Nous avons généré un anticorps polyclonal dirigé contre le site de phosphorylation putatif et avons révélé que non seulement cet évènement de phosphorylation se produit dans plusieurs types de cellules en réponse au traitement à l'angiotensine II (AngII), mais que l'activation de récepteurs multiples, comprenant un non-RCPG, le récepteur du facteur de croissance de l'épiderme, induisait la phosphorylation sur l'unité β de AP-2. Mon travail, précédemment publié dans un manuscrit, a également révélé que la prévention de cet évènement de phosphorylation stabilise les complexes βarrestine/AP-2 de

façon significative. Bien que nos études initiales aient révélé que cette phosphorylation a peu d'impact sur l'endocytose des récepteurs, de nouveaux outils ont depuis été développés dans le laboratoire. Premièrement, nous avons généré un anticorps à chaîne unique qui peut être exprimé de façon intracellulaire et cible les phosphosites, et avons démontré que la liaison à ce résidu phosphorylé résulte dans un arrêt prolongé de l'endocytose. Nous avons également démontré que ce résidu tyrosine phosphorylé est un motif putatif de liaison pour certaines protéines contenant un domaine SH2 étant donné que trois de ces protéines sont capables de se lier à une β2adaptine phosphorylée.

Enfin, en raison de la nature dépendate à βarrestine de cet évènement de phosphorylation, nous avons characterise quatre analogies de AngII avec une seule substitution d'acide amine dans leur sequence octapeptidique. Nos conclusions établissent une relation corrélative entre la force d'avidité de βarrestine pour son récepteur et le niveau d'activation de la kinase ERK suite aux signaux extracellulaires. Vraisemblablement, les différences d'avidité modifient le trafic du récepteur dans son destin vers le recyclage ou la dégradation. Par ailleurs, nous établissons que la conformation de βarrestine peut altérer les voies activées en aval du récepteur, entraînant d'autres effets cellulaires comme la croissance cellulaire ou la migration. Ces résultats mettent en évidence l'importance de la régulation par ces adaptateurs de l'endocytose sur le destin du RCPG, non seulement par leur rôle dans l'internalisation, mais aussi par leur propre potentiel de signalisation.

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This thesis is written in manuscript form as permitted by the McGill University Faculty of Graduate Studies and Research. It is composed of three separate manuscripts, as listed below, with the contribution(s) of each author. A fourth manuscript can be found in the appendix.

B. Zimmerman B, M. Simaan, M.H. Lee, L.M. Luttrell, S.A. Laporte.

c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway. *Cellular Signaling*. 2009 Jan;21(1): 103-10.

In this study, the candidate performed all the experiments included in the manuscript, performed data analysis and wrote the manuscript. M. Simaan helped in the design of the study and in editing the manuscript. MH. Lee and LM. Luttrell were responsible for the generation of a doxycycline inducible cell line for the knockdown of βarrestin that was used in the study by the candidate to demonstrate the βarrestin dependency of the observed effect. S.A. Laporte designed the study, analyzed the data and wrote the manuscript.

B. Zimmerman, Y. Namkung, E. Chien, S.A. Laporte.

A functional role for Y737 of β_2-adaptin in the signalling and endocytosis of the AT1R revealed through a single chain antibody. **In preparation**.

In this study, the candidate designed the experiments, performed many of the experiments excluding those listed below, analyzed the data and wrote the manuscript. Y. Namkung generated the DNA constructs to produce the single chain antibodies, and was involved in their validation. E. Chien helped in the characterization of the 10 monoclonal antibodies that were generated, which was essential in the determination of which antibodies should be selected for single chain antibody development. S.A. Laporte was responsible for the study design and wrote the manuscript.

B. Zimmerman, A. Beautrait, B. Aguila, R. Charles, E. Escher, A. Claing, M. Bouvier, and S.A. Laporte.

Biased Signalling by angiotensin analogs leads to differential βarrestin-dependent cellular responses. **Under review – Science Signalling.**

In this study, the candidate designed the experiments, performed many of the experiments excluding those attached to the alternate authors, was responsible for data analysis and wrote the manuscript. A. Beautrait and M. Bouvier's lab designed, performed and interpreted the BRET experiments. B. Aguila is a post-doctoral trainee in S.A. Laporte's lab who performed the FRAP experiments and some confocal microscopy experiments for figure 2. R. Charles is a Master's student in A. Claing's laboratory who performed the cell migration experiments in Boyden chambers. E. Escher was responsible for the synthesis of the angiotensin II analogs used in the paper. S.A. Laporte was involved in the study design and wrote the manuscript.

Abbreviations

7TM	Seven-transmembrane
AAK	Adaptin-associated kinase
AC	Adenylyl clyclase
ACEi	Angiotensin converting enzyme inhibitors
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AngII	Angiotensin II
AP-2	Adaptor protein 2
ARB	Angiotensin II receptor blocker
ATP	Adenosine triphosphate
AT1R	Angiotensin II type I receptor
AT2R	Angiotensin II type II receptor
βARK	β-adrenergic receptor kinase
cAMP	Cyclic adenosine monophosphate
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CTX	Cholera toxin
DAG	Diacyl glycerol
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
G protein	Guanine nucleotide-binding protein
GABA	Gamma amino butyric acid
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase

GTP	Guanosine triphosphate
IP_3	Inositol triphosphate
Jak2	Janus kinase 2
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PI-4,5-P_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PTH1R	Parathyroid hormone receptor 1
RGS	Regulators of G protein signalling
scFv	Single chain variable fragment
SII	Sar^1,Ile^4,Ile^8-AngII
V2R	V_2 vasopressin receptor

Table of Contents

APPENDICES

LIST OF FIGURES AND TABLES

CHAPTER 1 – Introduction

CHAPTER 2 – c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway

Chapter 1

Introduction

1. G protein-coupled receptors : an overview

The ability of an organism to respond to its extracellular environment is a key element in its survival and evolution. This extracellular information is translated into an intracellular response, allowing cells to acclimate through cellular processes of second messenger production, gene transcription and protein synthesis among others. Cell surface receptors represent a major component of how organisms respond to extracellular stimuli, with the most common family of membrane-bound receptor being the G protein-coupled receptors (GPCRs). The GPCR superfamily is composed of over 800 genes encoding seven-transmembrane (7TM) receptors that control a plethora of biological responses including but not limited to, neurotransmission, hormonal control of most physiological processes, taste, smell, vision and pain [1]. 7TM receptors or GPCRs are able to have such diverse roles as they are sensitive to neurotransmitters, ions, amino acids, hormones, growth factors, light, or odorant molecules. As such, much research for therapeutic development has focused on these receptors as they are targeted by 30-50% of therapeutic agents currently on the market [2]. 7TM receptors are generally referred to as GPCRs because the majority interact with one or more members of the intracellular guanine nucleotide-binding proteins (G proteins), specifically, the heterotrimeric G proteins. Upon stimulation, these receptors are thought to undergo conformational changes that allow for the activation of the G protein through the release of guanosine diphosphate (GDP) and its replacement with guanosine triphosphate (GTP). Controversy exists on whether the receptor is pre-bound by the G protein or if the conformational change also allows for their recruitment, although this thesis will not address this issue [3, 4]. G protein activation leads to the propagation of different signalling pathways through various effectors [5]. These effectors generate second messengers, which in turn regulate a vast number of cellular processes including cell growth, migration and differentiation.

1.1 Structure of GPCRs

All GPCRs are composed of a single polypeptide chain that contains seven hydrophobic transmembrane domains. These transmembrane domains are structured in alpha helices oriented perpendicularly to the plasma membrane and are referred to as TM-I through TM-VII. The transmembrane units are connected by three intracellular (IL1, IL2 and IL3) and three extracellular (EL1, EL2 and EL3) loops [6] (Figure 1). The N terminal region is always located in the extracellular milieu and is a frequent target of glycosylation, an important process in GPCR maturation. In contrast, the C terminal region is exclusively found in the cytosol. Its length varies between receptors and is also a frequent target for post-translation modification, often phosphorylation. Recently, crystal structures of the β_1- and β_2- adrenergic [7-10], dopamine D_3 [11], adenosine [12, 13] and CXCR4 chemokine [12, 14] receptors have been solved and highlight how specific domains of the receptor may be involved in ligand binding and activation. Unfortunately, the receptor structure for the angiotensin II type I receptor (AT1R), the receptor of focus in this thesis, has yet to be reported.

1.2 Classes of GPCRs

Despite their lack of sequence homology, GPCRs can be subdivided into three main structural families; A, B and C (Fig. 2). The Class A or rhodopsin-like family is the largest of the three, with its members responding to a broad variety of stimuli including photons (rhodopsin), odorants, neurotransmitters, and hormones. Many of the Class A receptors share critical highly conserved residues, most notably the Asp-Arg-Tyr (DRY) motif. The DRY motif comprises both the ionic lock for Class A GPCRs, preventing their constitutive activity, as well as represents critical residues for efficient G protein coupling [15, 16].

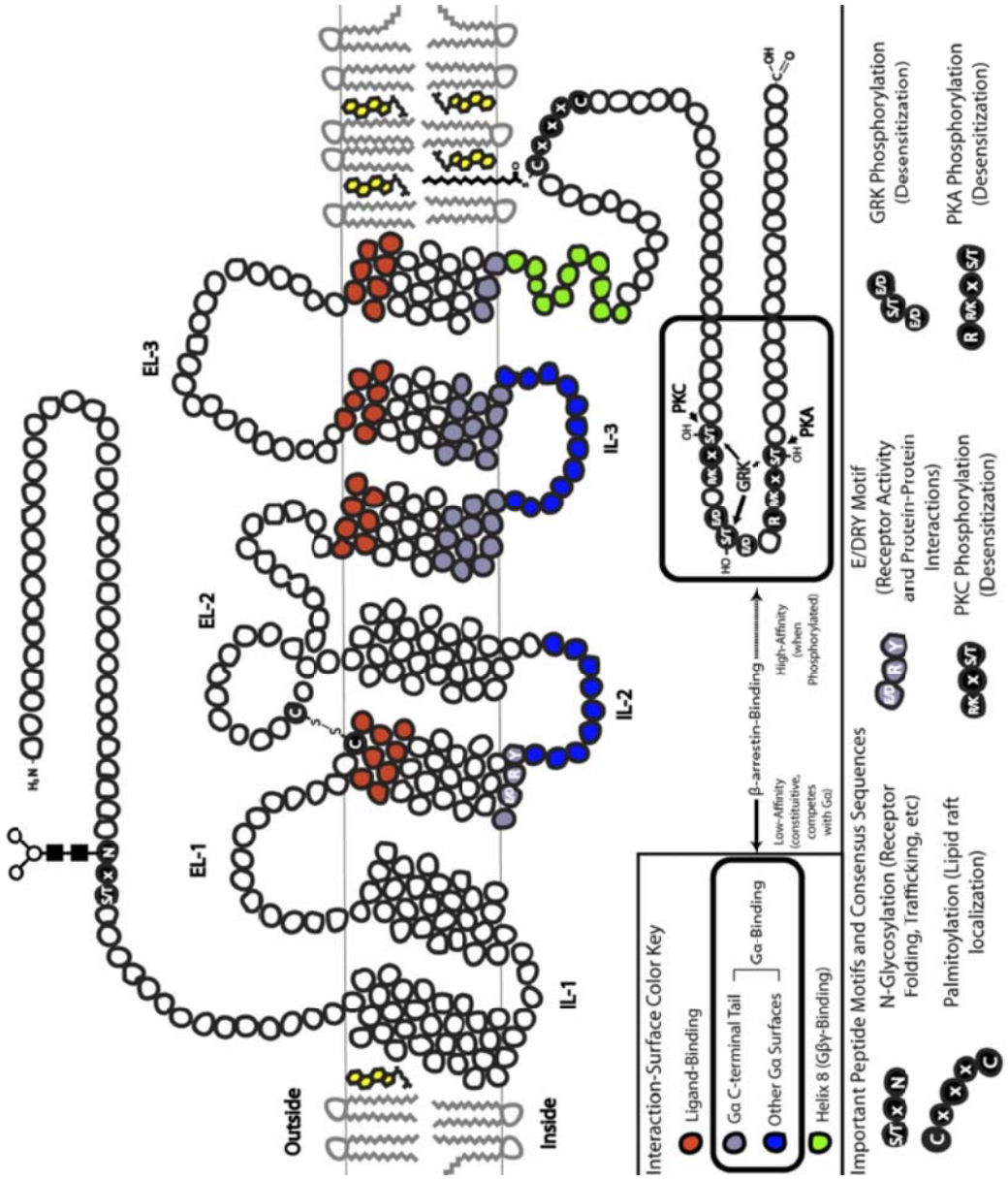

Figure 1. Schematic representation of the seven transmembrane spanning domains that form the transmembrane helices and the alternating extracellular (EL1-3) and intracellular (IL1-3) loops for a generic GPCR [17].

Class B receptors are known as the secretin receptor family, and are a small class of receptors (~25 members) that include the gastrointestinal peptide hormone receptor family, and the parathyroid hormone receptor among others. These receptors are primarily activated by large peptides, and contain a comparatively large cysteine-rich, N-terminal extracellular domain (~100 amino acids), which is critical for ligand binding [18]. While the DRY motif is absent in class B receptors, they possess their own set of conserved residues and motifs. A common characteristic of Class A and Class B receptors is found between the second and third extracellular loops, where a disulphide bridge is usually formed.

The Class C family is structurally distinct from the first two classes, as its members contain an extremely long amino-terminus compromised of 500-600 amino acids often referred to as the venus fly trap domain [19]. Unlike Class A and B GPCRs, the orthosteric site of ligand binding is found within the N terminal tail, rather than within the transmembrane helices. In addition, they possess a relatively shorter third intracellular loop that contains highly conserved residues of this family [20]. The prototypic member for this family is the metabotropic glutamate receptor, with the $GABA_B$ receptor and the calcium-sensing receptor making up some of the other members in this diverse class.

While these three form the main classes of GPCRs, there exists 3 minor families consisting of the class D, fungal mating pheromone receptors; the Class E, cyclic AMP receptors; and Class F, frizzled/smoothened receptors. Of these 3, Class F is the most biologically relevant as they are involved in Wnt and Hedgehog signalling to pathways that are critical during embryonic development. [21]

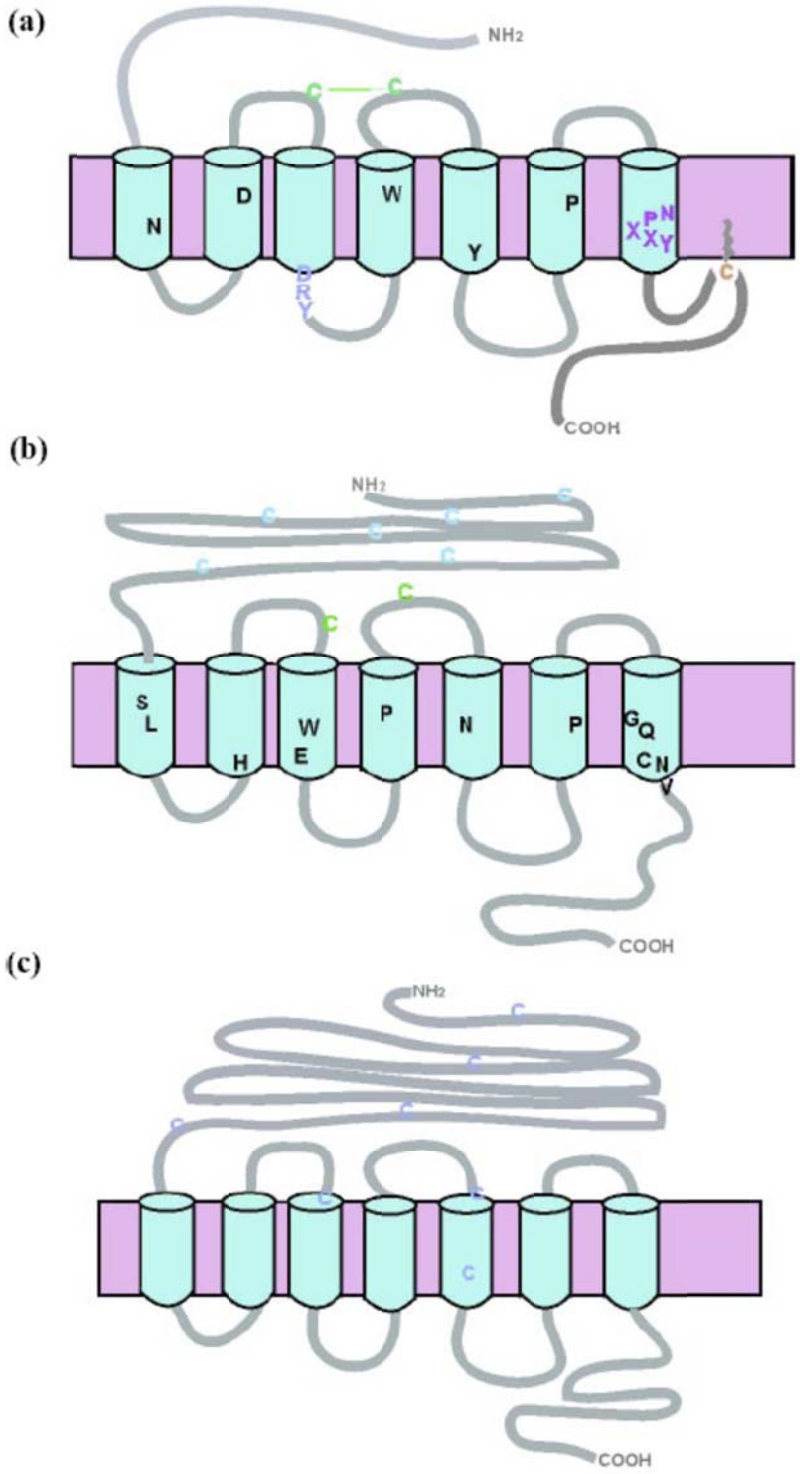

Figure 2. Schematic representations of the three major classes of GPCRs: Class A, Class B and Class C. Common motifs (purple), conserved residues (black) and cysteine residues connected by disulphide bridges (green) are shown [22].

1.3 GPCR ligands

GPCR ligands are chemically with similarly structured proteins being able to respond to light, odorants, hormones, and neurotransmitters among others. As GPCRs are important therapeutic targets, much research has gone into the development of compounds that target the receptors to either lead to their activation or inhibition. Pharmacologically, these compounds can be referred to in three main categories: agonist, antagonist or inverse agonist [23]. An agonist is a compound that activates the receptor. An antagonist has no biological effect at the receptor except to compete with the endogenous ligand in either a competitive or non-competitive manner for binding to the receptor. The concept of inverse agonism is a principle that requires basal signalling from the receptor population. In this case, an inverse agonist will bind to the basal active receptors and actually induce a conformational change that will lead to their inactivation. Physiologically, inverse agonism occurs at the melanocortin subtype 1 and 4 receptors with its endogenous ligands, agouti and agouti-related protein [24]. However, this is an over-simplified view of GPCR ligands, as an agonist or inverse agonist can have varying degrees of efficacy, which could lend it to being a partial activator or inhibitor of a pathway.

Furthermore, while the majority of compounds available currently target the orthosteric site (the binding site of the endogenous ligand), new compounds are being designed that act at other regions of the protein, which are referred to as allosteric ligands [25]. The pharmacology of this class of ligands is much more diverse, as they may be active in the absence of the endogenous ligand, *i.e* have intrinsic functionality of their own, or they may only be effective if an orthosteric compound is bound. They are referred to as allosteric modulators, and can moderate receptor activation in either a positive or negative manner [26-28].

1.4 G protein-dependent GPCR signalling

The discovery of the GPCR as we understand it today was a complex feat accomplished largely by Rodbell and Gilman for which they won the 1994 Nobel Prize in Physiology or Medicine. Rodbell pioneered this work based on his belief that like computer systems, biological systems must be composed of discriminators, transducers and amplifiers/effectors [29]. While both the concept of receptors and amplifier/effector were accepted notions in the late 1960s, it was unclear how this signal was actually being transduced, such that it could cross the plasma membrane. Rodbell's studies involved the hormone glucagon on rat liver tissue, where they believed the receptor was located. His group made a seminal observation that glucagon dissociated from the cell surface receptor in the presence of adenosine triphosphate, revealing the existence of an ATP-dependent transducer. He also noted that trace amounts of GTP accomplished the dissociation about one thousand times faster, and it was later deduced that ATP had no role in this process but that the ATP had contained trace amounts of GTP [30, 31]. Further research determined that the GTP stimulated the activity of a guanine nucleotide protein (G protein) that led to the activation of amplifiers/effectors like adenylyl cyclase [32]. Therefore, the elusive transducer between the discriminator and the amplifier, the G protein had been discovered. Subsequent studies determined the nature of the G protein, which in this case was $G\alpha_s$, and revealed that the G protein is a heterotrimer composed of an α, β, and γ subunit [33].

The exact mechanism of GPCR activation remains an open question with many different models having been proposed. The classical activation model (Fig. 3) implies that upon agonist binding, the GPCR will undergo a conformational change within the 7TM regions, allowing for the association of the heterotrimeric G protein and the exchange of GDP for GTP. The nucleotide switch allows for the activation of the Gα subunit and the subsequent dissociation of the Gβγ dimer.

At this stage, both the Gα and Gβγ subunits are free to activate second messenger signalling (Fig. 4). The Gα proteins contain intrinsic GTPase activity, and thus with time will hydrolyze the GTP to GDP, reassociate with Gβγ, ending the activation phase. However, as the intrinsic GTPase activity is quite poor, GTPase-activating proteins (GAPs), in this case regulators of G protein signalling (RGS) proteins, accelerate GTP to GDP hydrolysis [34, 35]. Despite this seemingly clear model, many facets are contentiously debated, none more so than whether the physical dissociation between the Gα and Gβγ subunits or the G protein and the GPCR occurs [36]. Recent evidence points to the lack of physical dissociation of the G protein itself or the G protein from the receptor but instead that significant conformational changes occur between the complex that enables the recruitment and scaffolding of effectors that induce second messenger signalling [37, 38]. The nature of the second messengers activated is dependent on the type of G protein or accessory proteins coupled to the receptor. To date, 23 α, 6 β and 14 γ subtypes have been described. On the basis of sequence similarity, the Gα-subunit has been divided into four main families, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, with this classification defining both receptor and effector coupling.

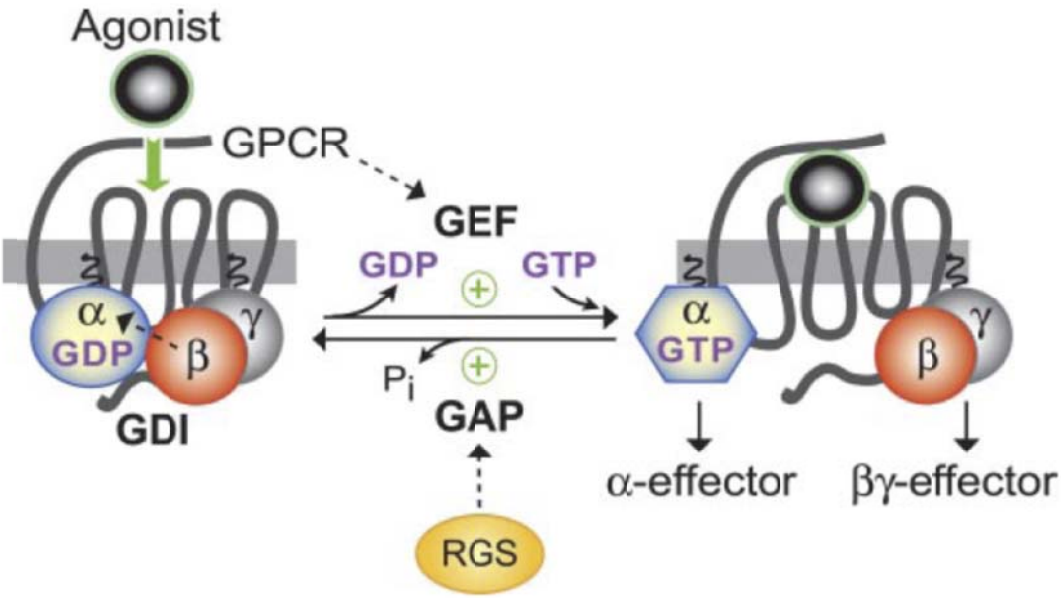

Figure 3. The regulatory cycle of a G protein. Activation of the GPCR induces the exchange of GDP for GTP with the aid of a GEF, which causes α and βγ dissociation and their ability to activate effectors. Eventually, the intrinsic GAP activity of the receptor, in addition to acceleration of GAP activity by RGS proteins, facilitates the termination of signaling by returning the G protein to the inactive GDP bound form [39].

1.4.1. $G\alpha_s$ signalling

Upon binding GTP, $G\alpha_s$ activates the enzyme adenylyl cyclase (AC), which catalyses the conversion of ATP to the second messenger, cyclic adenosine monophosphate (cAMP) [40]. Increased levels of cAMP activate protein kinase A (PKA), which is a serine/threonine kinase and an important metabolic mediator due to its regulation of glycolysis, gluconeogenesis and lipolysis [41]. Phosphorylation targets of PKA are diverse and include GPCRs, other proteins kinases like members of the mitogen-activated protein kinase (MAPK) cascade and transcription factors. More recent studies have revealed that $G\alpha_s$ can also directly activate some potassium channels, as well as the non-receptor tyrosine kinase, Src [42]. The classical GPCRd belonging to the $G\alpha_s$ family are the β-adrenergic receptors, while some of the dopamine and serotonin receptor family also couple to this G protein. Bacterial toxins from *Vibrio cholera* [cholera toxin (CTX)] are useful for studying the functional roles for $G\alpha_s$, as CTX catalyzes the adenosine diphosphate (ADP)-ribosylation of $G\alpha_s$ at an arginine residue within the GTP-binding domain. This event produces a constitutively active $G\alpha_s$, as the intrinsic GTPase activity of the protein is lost [43]. A second member of the $G\alpha_s$ family is the olfactory G protein, $G\alpha_{olf}$. This G protein is activated solely by olfactory GPCRs in response to odorant molecules and like $G\alpha_s$, leads to increases in cAMP concentration through activation of AC. However, here cAMP levels act to open cyclic-nucleotide-gated ions channels, allowing for depolarization of the sensory neuron, leading to an action potential that is transduced to the brain [44].

1.4.2. $G\alpha_{i/o}$ signalling

This family of G protein is particularly diverse with numerous subtypes. Despite this, most act in a similar manner, as they function in opposition of $G\alpha_s$, *i.e.* to inhibit adenylyl cyclase and decrease cAMP levels. $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_i$, $G\alpha_{o1}$ and $G\alpha_{o2}$ all inhibit AC, and have the potential to activate the extracellular signal-

regulated kinases (ERKs) and Src. However, other subtypes in the family are more diverse in their signalling potential, with $G\alpha_z$ upregulating calcium channels, $G\alpha_{t1/2}$ (transducin) downregulating potassium channels and activating phosphodiesterase, and $G\alpha_{gust}$ activating phosphodiesterase, in addition to potential inhibition of adenylyl cyclase [41]. Similar to $G\alpha_s$, this family is also sensitive to ADP-ribosylation by a toxin, in this case pertussis toxin (PTX) from *Bordetella pertussis* [45]. However, in this case ADP-ribosylation by PTX occurs on a cysteine residue and prevents the interaction of the G protein with the receptor rendering it inactive, as it cannot exchange from the GDP to GTP bound form.

1.4.3. $G\alpha_{q/11}$ signalling

The $G\alpha_{q/11}$ pathway is the classical pathway activated by calcium-mobilizing hormones. Stimulation of $G\alpha_{q/11}$-coupled receptors results in the activation of phospholipase C (PLC), which in turn cleaves the membrane bound phospholipid, phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) to produce two signalling second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [46]. IP_3 acts on its own receptor on the endoplasmic or sarcoplasmic reticulum, resulting in the opening of calcium channels and a dramatic flux of calcium ions into the cytosol [47]. Physiologically, increased calcium concentrations alone can result in muscle contraction through binding to myosin, in addition to being involved in the secretory pathways in some cells and in neurotransmitter release in neurons [48-50]. DAG, in concert with elevated calcium, activates protein kinase C (PKC) [50]. Like PKA, PKC is a serine/threonine kinase with a diverse array of targets and physiological roles. PKC signalling has been shown to regulate muscle contraction, neuronal excitement, cell growth and hematopoiesis [51-53]. PKC can be subdivided into three groups; classical, novel and atypical [54]. The classical group of PKCs (α, β1, β2 and γ) are involved post-$G\alpha_{q/11}$ activation as they require both elevated calcium levels and DAG to be

stimulated. Novel PKC isoforms do not require calcium, whereas atypical isoforms do not require calcium or DAG, and thus are less likely to be activated as a result of $G\alpha_{q/11}$ signalling [55]. Unlike $G\alpha_s$ and $G\alpha_{i/o}$, there are no toxins that inhibit $G\alpha_{q/11}$ signalling. Recently, a synthetic inhibitor, YM-254890, was developed by Astellas Pharma inc. in Japan, however, they are not releasing the compound for public use due to its apparent complicated synthesis [56]. This class of G protein signalling is of particular interest for this thesis, as the receptor of focus, the AT1R primarily is $G\alpha_{q/11}$-coupled.

1.4.4. $G\alpha_{12/13}$ signalling

This group of G proteins was discovered through comparative sequence analysis with other G proteins [57]. While $G\alpha_{12/13}$ is known to activate the Rho family of GTPases, $G\alpha_{12/13}$ signalling is considerably less understood compared to the other members of the Gα family. The involvement of guanine exchange factors (GEFs) and GAPs like RGS proteins play a larger role in the regulation of this class of G protein. $G\alpha_{13}$ directly interacts with p115-RhoGEF, and both $G\alpha_{12/13}$ proteins have relatively slow intrinsic rates of GTP hydrolysis, making GAP recruitment more critical in their regulation [58]. Changes of cellular morphology, alterations in vesicular trafficking, and cell polarity as a result of cytoskeleton reorganization are primary roles associated with Rho family activation [59]. Exactly how $G\alpha_{12/13}$ proteins function remains unclear as they promote both cell growth and apoptosis [60].

1.4.5. Gβγ signalling

Gβγ-subunits are tightly bound through noncovalent hydrophobic interactions and as such, signal as a single entity [61]. Their interaction affinity is so high that tryptic cleavage of the proteins does not lead to their dissociation; separation is only achieved with denaturating agents [62]. Despite the numerous Gβ and Gγ subtypes, which theoretically could produce over 80 different combinations of βγ complexes, certain βγ dimers are not possible, while others are highly unfavourable [63]. Nevertheless, many different dimers do form

between these proteins, leading to an extremely diverse makeup of the heterotrimeric G protein. While Gβγ was initially thought to have no role in signalling other than scaffolding Gα and keeping it in an inactive state by inhibiting GDP dissociation, it is now known to have at least as many interactors as Gα [64-66]. While the field is still undergoing development, the Gβγ heterodimer has been shown to couple directly to PLCβ, potassium channels, calcium channels, AC, phosphatidylinositol 3-kinase (PI3K), phospholipase A_2, the calcium ATPase transporter as well as proteins containing pleckstrin homology (PH) domains [67-72]. The coupling to AC is slightly complicated as some isoforms are activated while others are inhibited, thus the effect of Gβγ signalling on cAMP levels will depend on the AC isoforms expressed in the specific cell type. Perhaps the most important interacting-proteins in terms of GPCR signalling are G protein-coupled receptor kinase 2 and 3 (GRK2/3), which bind to Gβγ through their PH domains [73]. Recruitment of GRK leads to inactivation and desensitization of a GPCR, which will be discussed further in section 2.1.2.1.

1.4.6. Multiple G proteins at the same receptor

While understanding the biological role of receptors would be simpler if they were coupled to only one class of G protein, there is substantial evidence for multiple G protein-coupling. Numerous GPCRs have demonstrated the ability to couple to two or more classes of G protein, in some case working in direct opposition to one another. For instance, the β_2-adrenergic receptor stimulates both $G\alpha_s$ and $G\alpha_i$, which have opposite effects on AC activation. Therefore, in absence of compartmentalization, receptor signalling must be more complicated than simple activation of the receptor; otherwise there would be potentially no net effect. The same is true for the α_2-adreneric receptor, as it is sensitive to both cholera and pertussis toxins [74]. As such, we must take care in targeting specific GPCRs as the outcome in their activation or inhibition may not be as predictable as we would like or expect.

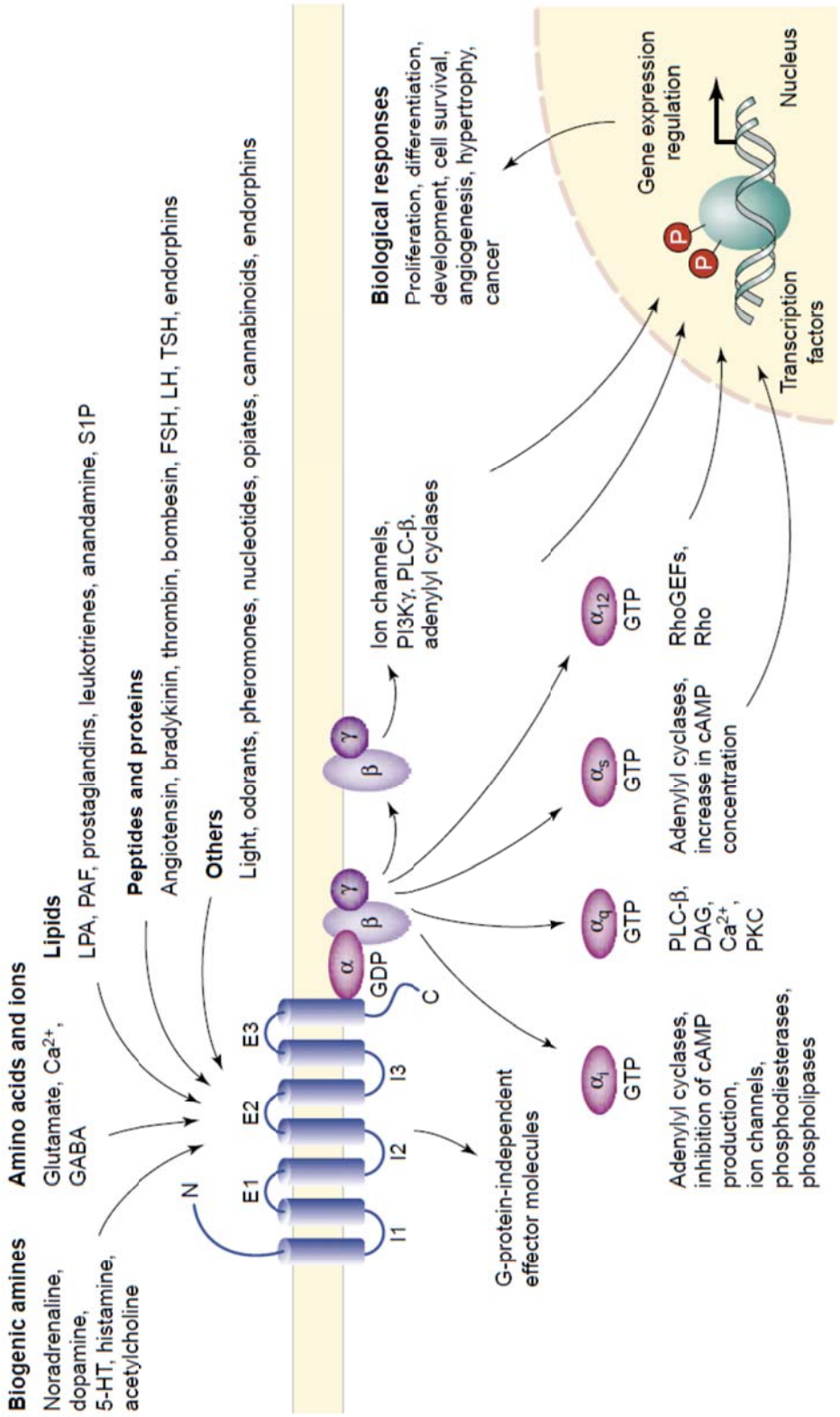

Figure 4. Schematic representation of the different forms of GPCRs signalling, the ligands and outcomes associated with them. [75]

2. Receptor desensitization and internalization

Following GPCR activation, an inactivation process commences, which consists of the desensitization and internalization of the receptor. This event serves to control the responsiveness of GPCR signalling by dampening the potential output, especially in instances of prolonged stimulation [76]. In fact, if receptor stimulation occurs at abnormally high levels, a third mechanism involving receptor downregulation can transpire, effectively decreasing the total receptor level [77].

2.1 GPCR desensitization

GPCR desensitization involves placing the receptor in an inactivated state where it can no longer propagate signal transduction from its initial stimulation, while also preventing further ligand-mediated activation. Essentially, the receptors are in an "off" state while desensitized. This process involves multiple phases, including receptor phosphorylation on numerous serine/threonine residues. This phosphorylation is accomplished by both second messenger-dependent protein kinases like PKA and PKC, and by GRKs. Many GPCRs containing serine/threonine clusters in their third intracellular loops or in their C termini are targeted by these kinases; however, other mechanisms of desensitization also exist as some receptors lack these residues, or substitution or loss of these residues bears no consequence for receptor responsiveness [78, 79].

2.1.1. Heterologous desensitization

Desensitization mediated by protein kinases can be further subdivided into two main mechanisms, the first involving the second messenger-dependent protein kinases, PKA and PKC. As mentioned, these kinases transfer phosphate groups from ATP onto serine/threonine residues on the intracellular surface of the GPCR. The desensitization mediated by these kinases is referred to as

heterologous, as the target is not the receptor that is ligand-bound but actually receptors in the vicinity of another activated receptor [80]. As a result of this heterologous desensitization, hyporesponsiveness to ligand stimulation is usually observed due to the inhibition of receptors in their resting state. As discussed in the GPCR signalling section (Section 1.4), PKA and PKC are activated by cAMP and DAG/Ca^{2+}, respectively, and while being involved in desensitization, they also play significant roles in signal transduction. Occasionally, while the phosphorylation may appear to terminate receptor signalling, it may actually result in a switch in G protein-coupling. This shift has been demonstrated for both the β_2-adrenergic and prostaglandin I_2 receptor, where coupling switched from $G\alpha_s$ to $G\alpha_i$ [81, 82].

2.1.2. Homologous desensitization

In the 1970s, experimental evidence had accumulated pointing to the phosphorylation of ligand bound receptors, although the specific kinase(s) responsible for this event remained elusive. The kinase was first elucidated in 1978 for the rhodopsin receptor, and thus termed rhodopsin kinase [83]. Almost 10 years would pass until the kinase for a second GPCR was discovered, in this case the β_2-adrenergic receptor, and therefore it was named β-adrenergic receptor kinase (βARK) [84]. Eventually, it was realized that these two kinases belonged to the same family, which is actually composed of seven members, and are now referred to as GRKs. GRK-mediated desensitization is defined as homologous, as it only affects agonist-occupied receptors [85]. Not only does this phosphorylation prevent further receptor activation, it also terminates the ongoing G protein signalling, which is primarily accomplished through the recruitment of an inhibitory protein, arrestin, which functionally uncouples G protein signalling,

2.1.2.1. G protein-dependent receptor kinase

As mentioned, GRKs phosphorylate GPCRs at serine and threonine residues localized to the third intracellular loop and the C-terminal tail [86]. To

date, no consensus motifs for GRK phosphorylation have been identified; therefore determination of target residues in a receptor must be tested by mutagenesis or through phosphorylation-sensitive mass spectrometry. As a result, while GRKs are known to phosphorylate the majority of GPCRs, the key regulatory sites on most GPCRs remains unknown. Seven members of the GRK family have been identified with all containing three distinct domains critical for their function: a kinase domain, an N-terminal RGS domain, and a C-terminal domain responsible for targeting to the plasma membrane [87]. The RGS domain gives GRKs the added ability of terminating active G protein signalling, making them extremely efficient terminators of GPCR activation. While some studies have begun to differentiate the roles of the various GRK family members, in many cases GRKs are functionally redundant. Instead, it is their tissue distribution and level of expression that largely determines, which GRK member will be predominantly involved. Nevertheless, some evidence has pointed to GRK2/3 being more involved in receptor desensitization, whereas GRK5/6-dependent phosphorylation directs some receptors towards βarrestin-dependent ERK activation [88]. GRK activity is modulated by various mechanisms including binding the activated GPCR, interactions with accessory proteins, as well as their recruitment to or association with the plasma membrane. The C-terminal domain, *i.e.* the domain responsible for localization to the plasma membrane varies between the seven GRKs. GRK1/7 contain motifs that promote their farnesylation and carboxymethylation [89], GRK2/3 have binding domains for Gβγ, GRK5 uses a phospholipid binding domain, and GRK4/6 have motifs for palmitoylation. An aspect that further complicates homologous desensitization involves PKA- and PKC-depedent regulation of GRK function [90].

The seven GRK members can be divided into three sub-families according to their sequence similarity; GRK1/7, GRK2/3, and GRK4/5/6 [90]. The GRK1/7 group is localized exclusively to the retina with GRK1 being the originally identified rhodopsin kinase [91]. Translocation to the plasma membrane occurs

as a result of farnesylation and is essential for receptor phosphorylation [92]. The protein recoverin, whose distribution of expression nearly mimics that of GRK1/7, serves to inhibit GRK1/7 function in a Ca^{2+} manner, by preventing GRK's interaction with the activated receptor [93]. The GRK2/3 group was originally known as βARK1/2, as its first substrate was the β_2-adrenergic receptor. These two GRKs have a Gβγ binding domain in their C-termini and thus are always in close proximity to GPCRs [94]. They also contain PH domains, which facilitates their binding to plasma membrane bound PI-4,5-P_2 [95]. This group of GRKs is regulated by other protein kinases, as MAPKs decrease their activity, PKA and PKC potentiate it and Src and MAPKs can target these GRKs for protein degradation [96, 97]. Finally, the GRK4/5/6 subfamily is always located at the plasma membrane due to the palmitoylation of GRK4/6 and phospholipid binding domain of GRK5 [98]. All members in this family bind to calcium and calmodulin, which results in their inhibition, as they are no longer able to efficiently bind to the receptor or phospholipids [99]. While GRK5 and GRK6 expression is largely ubiquitous, GRK4 expression is mainly confined to the testis. [100]

While GRK phosphorylation can result in the desensitization of a GPCR alone, its main role in desensitization is the creation of a high affinity binding site on the GPCR for the recruitment of a cytoplasmic accessory protein. This protein was discovered to be part of the arrestin protein family, which function not only to desensitize the receptor but possess roles as internalization adapters and signalling scaffolds. Arrestin was first identified in the retina as a 48kDa protein bound to the rhodopsin receptor [101]. Its recruitment and binding to the receptor is usually dependent on the receptor's phosphorylation status, and recently, it was determined that the sites of phosphorylation can determine arrestin's role [102]. Impairment of phosphorylation by truncation of the GPCR C-terminus or substitution of the key serine/threonine residues leads to

diminished arrestin binding, as seen with rhodopsin, the bradykinin B_2 receptor and the AT1R [103-105].

2.1.3. Arrestin family proteins

Arrestins are divided into two main classes based on their cellular distribution, visual and nonvisual arrestins. In mammals, there are four arrestin proteins described to date, with two (visual arrestin – arrestin1 and cone arrestin – arrestin 4) being restricted to the retina for phototransduction [106]. The other two arrestin proteins are ubiquitously expressed, arrestin2 and arrestin3. They regulate the signalling and internalization of many GPCRs [107]. Arrestin2 and arrestin3 were termed βarrestin1 and βarrestin2 respectively, as they were discovered for their functional regulation of the β_2-adrenergic receptor [108]. These two arrestins share almost 80% amino acid sequence identity, and are thought to be redundant in the majority of situations [109]. For example, βarrestin1 or βarrestin2 knockout mice are healthy and viable, whereas double knockout are mice die during embryonic development [110, 111]. However, there are instances where unique roles from βarrestin1 or βarrestin2 have been revealed [112, 113]. In fact, in chapter 5 of this thesis, we find that only βarrestin2 is involved in the cell growth of rat vascular smooth muscle cells (VSMCs), while βarrestin1 appears to have an inhibitory role on their migration.

2.1.3.1. βArrestin interacting proteins

Since the discovery of βarrestin1/2, much research has focused on their interacting proteins. The number of potential interacting proteins exploded in 2007, when Lefkowitz's group published a proteomic study describing the βarrestin interactome [114]. This study described 71 and 164 unique interactors for βarrestin1 and βarrestin2, respectively, in addition to another 102 proteins, which bound to both. While many of the targets found need to be more thoroughly validated, prior to this study many protein interactions had already been confirmed. Select examples of validated protein partners include the E3

ubiquitin ligase, MDM2, small GTPases like ARF6, members of the MAPK family like ERK and JNK and of particular interest to our lab, endocytic proteins, clathrin and the β-subunit of AP-2 [115-120]. More recently, βarrestins have been shown to homo- and hetero-oligomerize, although no clear functional relevance has been demonstrated [121]. A potential explanation for this oligomerization involves the ability of GPCRs themselves to form dimers or oligomers, thus it may be beneficial for the arrestins to be recruited to the receptor in a similar manner.

2.1.3.2. βArrestin-mediated signalling

While βarrestin was originally described as a desensitizer and endocytic adaptor, it is now appreciated for its ability to act as a signalling scaffold, inducing the activation of numerous signalling pathways. In addition to scaffolding the different families of MAPK (ERK and JNK), βarrestin also binds and activates the Src family of kinases, the ARF6-ARNO complex, dishevelled 2, phospholipase A_2 and AKT [122-126]. Furthermore, it activates numerous other factors that lead to the termination of G protein signalling including diacylglycerol kinase [127] and cAMP phosphodiesterases [128, 129]. Other signalling paradigms affected by βarrestin largely depend on the internalization of the GPCR and its stability with βarrestin. It is apparent that βarrestin is no longer recognized solely as a desensitizer of GPCR action (Fig. 5).

2.1.3.3. Differential affinities of GPCRs for the βarrestins

Following agonist-mediated activation of a GPCR, GRK phosphorylation leads to the recruitment of βarrestin from the cytosol to the receptors at the plasma membrane [130, 131]. However, with the generation of green fluorescence protein (GFP)-tagged versions of βarrestin1 and βarrestin2, it became apparent that

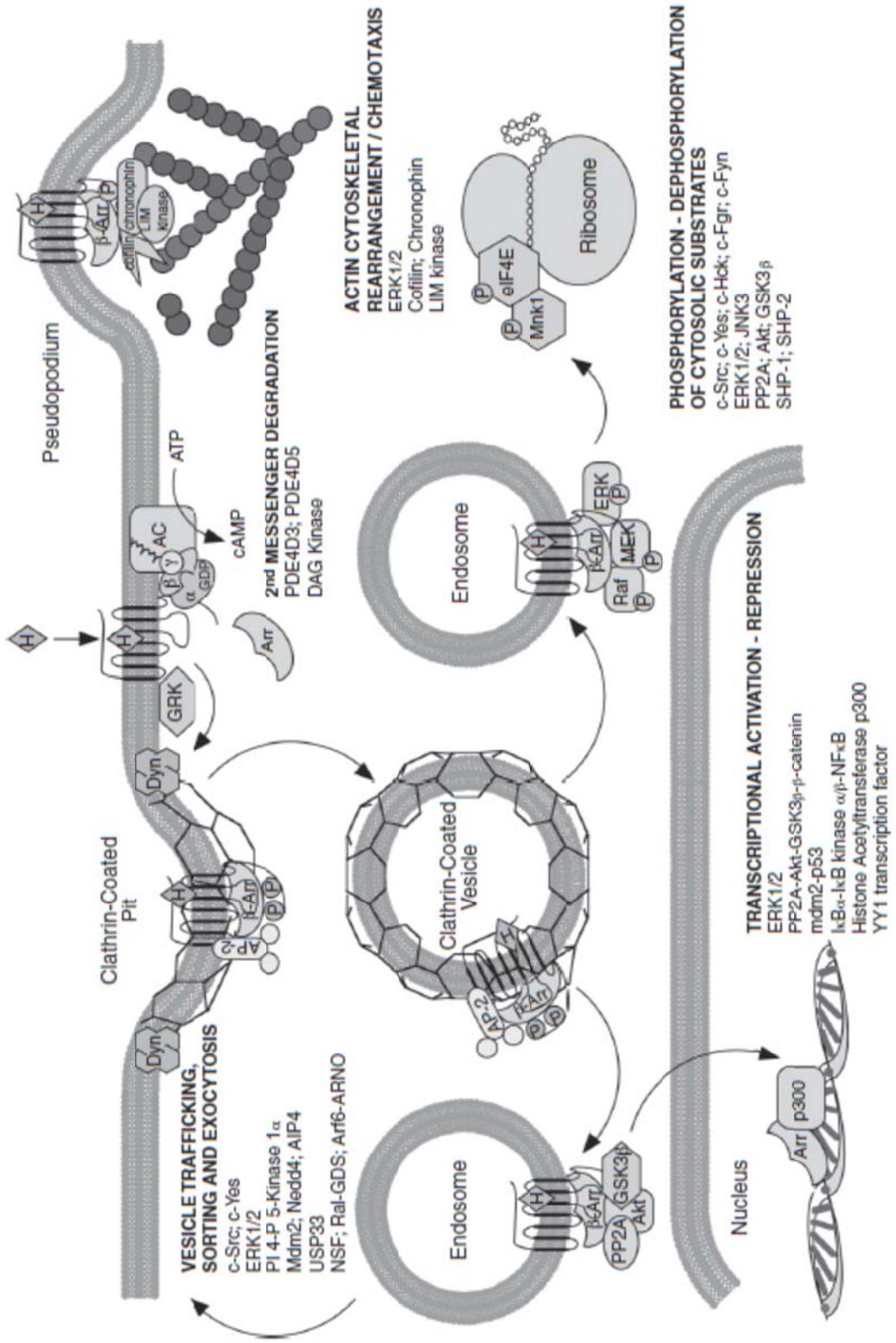

Figure 5. The various βarrestin-dependent signalling events that can occur [122].

GPCRs could be divided into two sub-classes based on their arrestin binding avidity. The first class of receptors, referred to as Class A receptors (not to be confused with Class A defined by the structural similarities between the GPCRs), include the prototypical β_2-adrenergic receptor and were demonstrated to have a relatively higher affinity for βarrestin2 compared to βarrestin1 [132]. However, what makes this class much more easily definable compared to class B receptors, which have equally affinities for the βarrestins, entails their relative weak affinity for arrestin in general, leading to the rapid dissociation of βarrestin from the receptor following internalization, and rapid recycling to the plasma membrane [133, 134]. Conversely, class B receptors form a relatively strong interaction with both βarrestins such that these receptors internalize with βarrestin and remain associated in endosomes with them for extended periods of time, leading to slow or absent recycling of the receptor. A prevailing explanation for this disparity in affinity lies with the density of GRK-mediated phosphorylation with more sites of phosphorylation believed to lead to stronger binding [135]. The difference in receptors affinities also appears to lie within βarrestin itself, as C-terminally truncated βarrestins (βarrestin1-383X or βarrestin2-381X) bind to Class A receptors with similar affinity to wildtype βarrestins at Class B receptors [103, 136]. This result suggests that βarrestin can regulate its own binding, by possibly masking or preventing high affinity binding through its C-terminal domain. Our lab has actually proposed a third class of receptor, Class C, which would fall in between Class A and Class B in terms of βarrestin affinity [137]. While there is no preference for βarrestin1 or βarrestin2 and the receptors internalize with βarrestin like class B receptors, following entry into early endosomes, the receptors (e.g. the bradykinin B_2 receptor or neurokinin type 1 receptor) dissociate from βarrestin and recycle rapidly to the plasma membrane [137, 138].

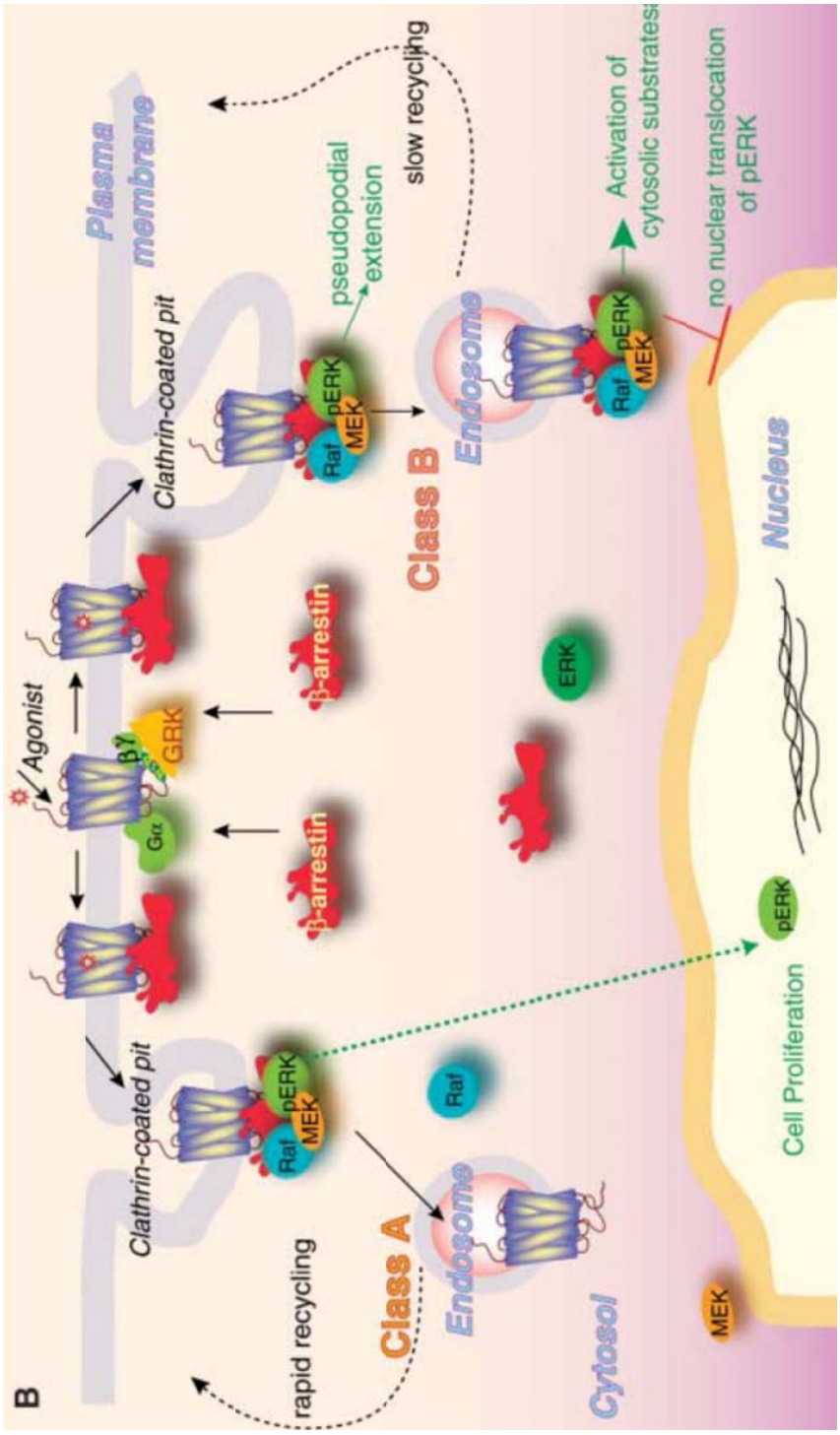

Figure 6. βarrestin-dependent internalization of a GPCR. GPCRs following endocytosis can either recycle rapidly (class A) due to their poor affinity for βarrestin, or can remain in endosomes for extended periods of time (class B) [139].

2.2 GPCR internalization

Clathrin-mediated endocytosis is a well-described mechanism for the entry of receptors and other cargoes to enter into cells. It involves the recruitment of soluble clathrin from the cytoplasm to the plasma membrane via accessory proteins that lead to clathrin-coated pit formation. The two main adapters associated with clathrin-dependent internalization of GPCRs are the adapter protein 2 (AP-2) and βarrestin [140]. Other mechanisms for GPCR internalization do exist [141, 142]. The main alternative pathway uses the protein caveolin, while a less understood pathway has adapters that have yet to be defined [143].

2.2.1. Clathrin-dependent internalization

The majority of GPCRs use clathrin to internalize, whether it is to be resensitized and recycled, or if it is to be sent for proteolytic or lysolytic degradation [144]. The conventional mechanism for this pathway first involves GRK-mediated phosphorylation of the GPCR, to enhance βarrestin binding. While βarrestin serves as a negative regulator of G protein-dependent signalling, it also acts as a scaffold for two components of the clathrin internalization machinery, AP-2 and clathrin [120]. Usually, AP-2 plays a pivotal role in clathrin-coated pit (CCP) formation, as it is responsible for the assembly of clathrin monomers, into the triskelia, in addition to the selection of cargo that will be internalized [145, 146].

2.2.1.1. Clathrin

The protein clathrin and its characteristic structure were originally identified by electron microscopy [147]. Clathrin, when monomeric, is in the form of a triskelia, composed of three heavy and three light chains that can oligomerize to form a polyhedral lattice [148]. In cells, this lattice surrounds a vesicle and is referred to as a clathrin coat. While clathrin can at times form this

oligomeric structure spontaneously, it happens much more readily in the presence of adaptors [149, 150].

2.2.1.2. Clathrin adaptor protein family

Internalization adapters usually serve to link the cargo with the endocytic machinery, to facilitate their endocytosis. There are numerous accessory factors to the clathrin-mediated internalization process with many of them acting as direct clathrin adaptors [151]. The AP-2 complex was the first protein to be characterized as a clathrin adaptor [152]. AP-2 belongs to a highly conserved protein adaptor family with three other members AP-1, AP-3 and AP-4, which display differential cellular localizations (Fig. 6) and therefore functions [153]. All members of the family are heterotetrameric proteins consisting of two large subunits (α, β_2 in AP-2), one medium subunit (μ_2) and one small subunit (σ_2) (Fig. 7). The two large subunits have two main structural domains, the core or truck domain and the appendage or ear domain, which are connected by a flexible, protease-sensitive linker, or the hinge.

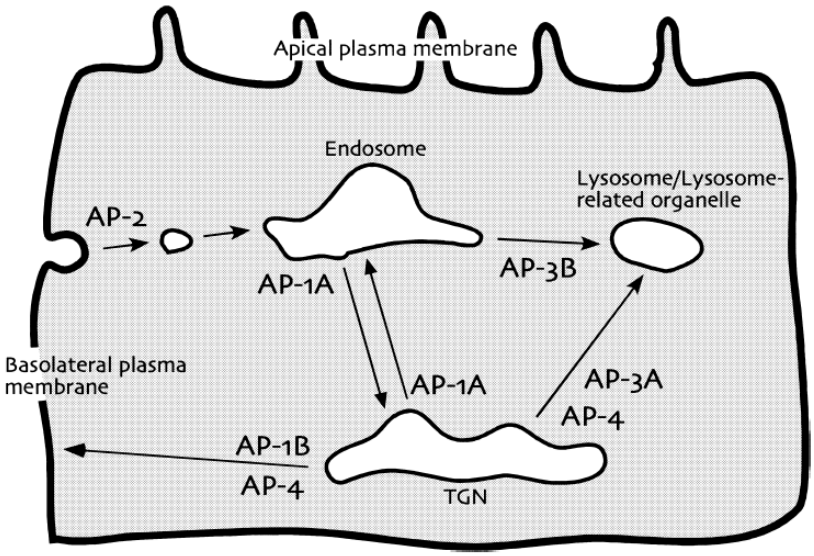

Figure 7. Localization of different AP complex family members. While AP-2 is concentrated at the plasma membrane, the other AP members are localized in endosomes, lysosomes or the Trans Golgi Network. [154]

As mentioned, endocytic cargo and the clathrin coat are bridged by AP-2, however, they also interact with numerous other proteins involved in clathrin-coated vesicle (CCV) formation. These accessory proteins form the dynamic network of protein-protein interactions that are required for the various steps of endocytosis. The subunits of AP-2 have distinct roles with the μ_2-subunit primary involved in cargo recognition through the NPXY motif [155]. These motifs also play a significant role in sorting post-endocytosis, as they can determine the final destination of the internalized cargo [156]. An additional role for this subunit pertains to its phosphorylation by adaptin-associated kinase (AAK), which phosphorylates μ_2-adaptin at threonine 156. This event is critical in proper receptor trafficking, as cargo bound to the μ_2-subunit cannot be released without T156 phosphorylation [157, 158]. The interaction of clathrin with AP-2 occurs through a clathrin box motif (LϕxϕD/E – ϕ = any hydrophobic amino acid) in the β_2-subunit (herein referred to as β_2-adaptin) found specifically in the hinge domain that links the truck and ear domains [159]. There are numerous other interactors with β_2-adaptin including βarrestin, epsin, AP180 and ARH [160-162]. The α-subunit also interacts with numerous proteins including clathrin, dynamin and Eps15 [163, 164], which is what makes AP-2 such a multifunctional adaptor. Loss of the α and μ_2 subunits by small interfering ribonucleic acid (siRNA) leads to the a block in clathrin-dependent endocytosis, however, knockdown of β_2-adaptin is insufficient, as it is replaced by β_1-adaptin in the heterotetramer [165]. Knockdown of both β subunits also leads to a block in endocytosis. AP-2 has also been demonstrated to be involved in embryogenesis as depletion of the complex in transgenic mice leads to embryonic lethality [166].

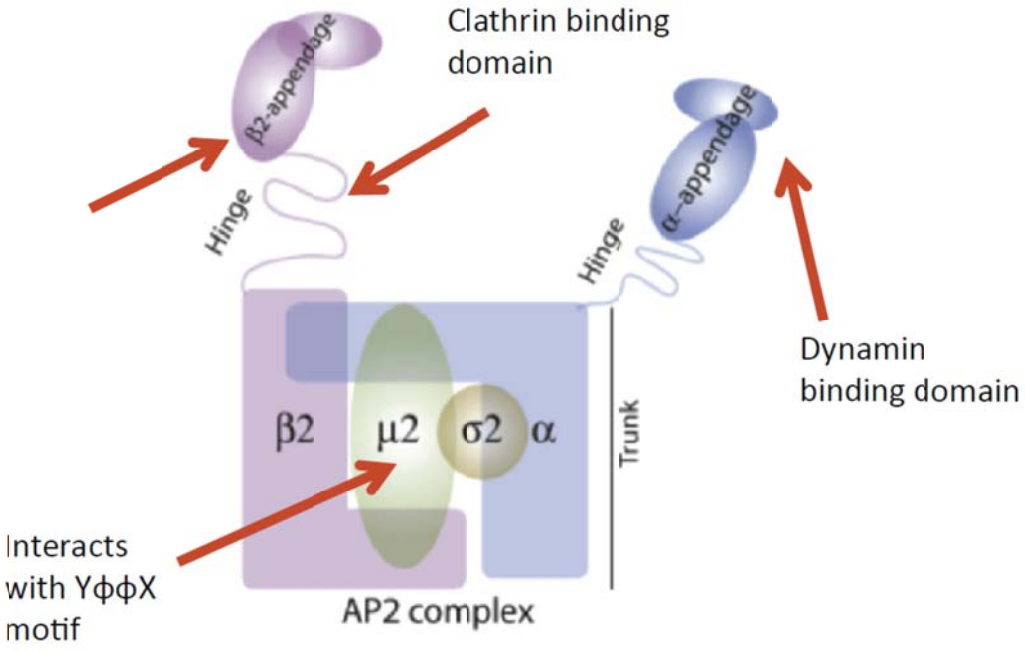

Figure 8. Representation of the heterotetrameric AP-2 complex. AP-2 binds to clathrin through its μ and α subunits [146].

2.2.1.3. βarrestin

βarrestin plays a significant role in GPCR endocytosis across numerous species including *Homo Sapiens, Drosophila melanogaster* and *Mus musculus [110, 112, 167].* Studies using antisense oligonucleotides to decrease βarrestin expression revealed βarrestin's involvement in endocytosis. These studies found that β_2-adrenergic receptor internalization was reduced when βarrestin2 was knocked down [168]. Further studies using mouse embryonic fibroblasts (MEFs) that lacked either βarrestin1 or βarrestin2 revealed differential roles for the two βarrestins in the internalization of different GPCRs [169, 170]. Multiple studies have demonstrated that besides βarrestin's main role in receptor desensitization, it also functions as an endocytic adaptor with its clathrin and AP-2 binding domains essential for receptor internalization [171, 172]. Indeed, truncation of βarrestin, where the β_2-adaptin binding domain is lost (βarrestin1-383X or βarrestin2-381X) can lead to trapping of the GPCR at the plasma membrane. Furthermore, expression of the C-terminal fragment of either βarrestin scavenges free AP-2 and clathrin, preventing their use in receptor internalization [173].

2.2.1.4. Src

Src, a member of the non-receptor tyrosine kinase family, can be recruited to GPCRs directly or through interaction with βarrestin [174]. The Src family of kinases consists of 9 members (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk) that contain similar structural elements and active sites. The key structural features to Src family kinases include a myristoylated N-terminal domain to facilitate localization to the plasma membrane, an SH3 domain responsible for recognizing proline rich regions, a SH2 domain to bind phosphorylated tyrosine residues, the kinase domain (SH1) and a C-terminal region [175]. Within the C-terminal region there are two key residues, Y418 and Y530, which control the activity of Src differentially, with phosphorylation of Y418 increasing Src activity,

and Y530 phosphorylation inhibiting Src entirely. Inhibition is achieved through self-binding, where the SH2 domain of Src potently recognizes this phosphotyrosine residue. Furthermore, Src possess a proline rich region in the SH1 domain, which allows for the binding of the SH3 domain, placing Src in an inactive state. Dephosphorylation of Y530 or competitive binding for the SH2 domain, in addition to competition for the SH3 domain allows for the activation of Src. Truncation of the N-terminal region until residue 249 generates an constitutively active Src protein called Src-SH1, as it lacks the autoregulatory domains [176]. K298 is the key residue in the active site of Src kinase and substitution of this residue with arginine or methionine generates a dominant-negative, kinase-dead Src protein, with the arginine substituted protein being the more stable [176].

While Src is primarily involved in signalling cascades, it also plays a role in the activation and regulation of the endocytic machinery [177-181]. Catalytically inactive Src proteins function as inhibitors of clathrin-mediated receptor internalization. Furthermore, Src phosphorylates a number of endocytic proteins including dynamin and GRK2 among others [182, 183]. In MEF cells lacking three members of the Src tyrosine kinase family, Src, Yes and Fyn, the β_2-adrenergic receptor cannot internalize [177, 183, 184].

2.2.1.5 Dynamin

The protein dynamin is an essential mediator of endocytosis, as it is responsible for the fission of the CCP from the membrane. Dynamin is a GTPase originally discovered in *Drosophila melanogaster*. This protein is temperature sensitive in *Drosophila*, as such elevation of temperature was found to prevent the endocytosis of CCPs, which resulted in their paralysis due to an inhibition of synaptic vesicle recycling [185]. While dynamin is relaxed in the presence of GDP, GTP hydrolysis by dynamin causes a large conformational change that results in the constriction of the underlying lipid layer [186]. Three isoforms of dynamin

have been found; dynamin II, which is ubiquitously expressed, dynamin I, localized to neurons and neuroendocrine cells and dynamin III, found primarily in the testes but also the heart, lung and brain [187]. The GTPase activity of dynamin is largely self-regulated, such that activation occurs as dynamin oligomerizes and reaches a critical mass. A secondary factor regulating its activity is PIP_2, which binds dynamin through its PH domain and aids in its localization to the plasma membrane [188]. The active site residue of the GTPase function of dynamin is K44, as substitution of this residue with alanine yields a dominant negative mutant that strongly impairs dynamin function [189]. Alternatively, substitution of K535 for alanine in the PH domain produces similar effects [190], although the K44A substitution is a more effective dominant negative. Another regulatory factor of dynamin function is phosphorylation by Src family kinases on two tyrosine residues (Y231/Y597). Substitution of these residues by phenylalanine prevents Src-dependent phosphorylation of dynamin, and has been shown to block β_2-adrenergic receptor endocytosis [181].

2.2.2. Caveolin-dependent internalization

Although the general model for GPCR internalization involves clathrin and βarrestin, some receptors continue to internalize even in their absence or inhibition. For instance, the 5-hydroxytryptamine 2A receptor ($5HT_{2A}R$), the M_1 and M_3 muscarinic receptors all continue to internalize in the presence of βarrestin C-terminal domain constructs that efficiently block clathrin-mediated endocytosis [191, 192]. Instead, it seems these receptors use an alternative pathway involving the internalization adaptor, caveolin. Caveolin is found is distinct structures from clathrin-coated pits called caveolae and are frequently found in lipid rafts [193]. While this pathway is distinct from clathrin-mediated endocytosis, it still relies on dynamin for fission from the plasma membrane [194]. Many GPCRs are located in caveolae based on electron microscopy, although it is not always clear that they remain within this region following

stimulation [195]. Indeed, many receptors internalize through both pathways, where inhibition of one pathway results in compensation by the other [196, 197]. While it is unclear the physiological consequences from using different routes of internalization, the cholecystokinin receptor undergoes lysosomal degradation following clathrin-dependent endocytosis, whereas following caveolin-mediated internalization, it does not [198]. However, this is not seen for other receptors, so it seems to require case by case analysis.

2.2.3. Clathrin- and caveolin-independent internalization

Receptor endocytosis is even more complex, as the inhibition of both clathrin- and caveolin-dependent pathways can fail to prevent receptor internalization. This phenotype has been demonstrated for numerous GPCRs including the M_2 muscarinic, *N*-formyl peptide and secretin receptors, although the exact mechanism of their internalization is still largely undefined [199-201].

2.2.4. The fate of internalized receptors

Following internalization, receptors can be targeted to specialized intracellular compartments, recycled back to the plasma membrane or processed for degradation. Multiple factors determine receptor trafficking including strength of association for βarrestin and phosphorylation status of the receptor. The affinity of the βarrestin for the receptor is not only regulated by the receptor and its phosphorylation but also based on the post-translational modifications that occur to βarrestin itself, in particular ubiquitination [202, 203]. While the βarrestin ubiquitination is dynamic, it has been demonstrated to be more transient for Class A than Class B GPCRs, and functions as a sorting signal for receptor degradation to lysosomes [204]. Further evidence demonstrating Class B GPCRs are more likely to undergo degradation based on their increased affinity for βarrestin stems from studies using chimeric receptors. Conventionally, the β_2-adrenergic receptor recycles rapidly, whereas the V_2 vasopressin receptor (V2R)

does not. However, if the C-terminal tail is switched between the two receptors, they switch classes and trafficking patterns [144]. Similar studies have been performed with multiple receptor combinations [205].

The purpose of GPCR internalization can be three-fold; the desensitization, resensitization or to allow intracellular signalling of the receptor. These processes are critical for the proper regulation of signalling within cells. Originally, internalization was thought to solely involve the desensitization or the receptor, as it appeared to result in the uncoupling of the receptor from its effectors [206]. As such, GPCRs were removed from the cell membrane to prevent receptor overstimulation and maintained in endosomes or targeted for degradation. The desensitization step as discussed above is largely controlled by GRK and βarrestin.

In order for the majority of GPCRs (excluding rhodopsin) to be resensitized, they require internalization into early endosomes where there is a high concentration of phosphatases [207]. Indeed, activated receptor isolated from the plasma membrane compared to early endosomes display increased levels of phosphorylation [208]. Receptors that resensitize rapidly dissociate from βarrestin as they internalize, which is thought to facilitate access of phosphatases to the phosphorylated receptor. An alternative form of cellular resensitization does not involve receptor recycling, but instead involves *de novo* synthesis of new receptors or the mobilization of intracellular spare receptors [209, 210].

Recently, it has become appreciated that GPCR internalization enables the receptor to engage in intracellular signalling, mostly through the βarrestin scaffolding [211, 212]. While βarrestin was originally described as a desensitization molecule, it is now clear that it plays a critical role in the outcome of GPCR activation. A key signalling paradigm that is propagated by βarrestin involves the activation of MAPK family including ERK and JNK [213]. This is achieved through βarrestin scaffolding, where it binds all three members of each

MAPK cascade. For instance, it is able to bind Raf, MEK and ERK, to mediate the sustained activation of ERK in the cytosol [213, 214], allowing the regulation of cell growth, division, differentiation and apoptosis via intracellular signalling. βarrestin-mediated activation of MAPKs leads to their retention in the cytosol, preventing the nuclear-mediated transcriptional effects of the kinases [215].

3. The angiotensin type I receptor

3.1. The discovery of Angiotensin II and the AT1R

Angiotensin was originally discovered based on its action as a pressor, and was called either hypertension or angiotonin [216, 217]. It was evident that a substance from the kidney, later determined to be renin, was responsible for the cleavage of a plasma protein substrate that resulted in the generation of this pressor compound [218-220]. The existence of two receptors for angiotensin only became clear at the end of the 1980s. They were eventually termed angiotensin type I and type II receptor (AT1R and AT2R) [221].

3.2. Angiotensin II

The octapeptide hormone, Angiotensin II (AngII – Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), the effector of the renin-angiotensin system has many physiological effects with all pointing towards a similar outcome, an increase in blood pressure. This increase is mediated through sympathetic activation, vascular smooth muscle cell contractility and vasoconstriction, water and salt retention both directly and through aldosterone release, and elevated vasopressin levels that also act to retain water (see figure 8) [222]. As such, pharmacological inhibition of AngII represents a popular mechanism for the prevention and treatment of hypertension and congestive heart failure. Two forms of inhibitors exist the first; angiotensin converting enzyme inhibitors (ACEis) prevent the formation of AngII for AngI by inhibiting angiotensin converting enzymes (explored in figure 9), whereas the second class of drugs are competitive inhibitors at the receptor, angiotensin receptors blockers (ARBs) [223]. While

ACEis are generally the preferred form of treatment, they have a specific side effect of dry cough, as a result of elevated bradykinin levels [224]. Bradykinin is also a substrate for the converting enzyme, and as such it is not metabolized if an ACEi is given. More recent compounds targeting the AT1R are attempting to act as partial agonists/antagonists at the receptor depending on the downstream pathway, which will be discussed further below [225].

As mentioned, AngII is an octapeptide hormone and much research has been dedicated to the structure-activity relationship of the different amino acids in its sequence. Of particular importance are residues Y4 and F8, which play significant roles in the maintenance of AT1R-mediated G protein activation [226]. H6 has been shown to be important for ligand binding and efficacy at the receptor, whereas Arg^2 is thought to be important for AngII receptor recognition. Finally, Asp^1 plays a minor role, although substitution with the synthetic amino acid, sarcosine, can yield a more stable AngII compound as it is no longer a substrate for the aminopeptidase, angiotensinase A [227]. Previously, it was thought that substitution of position 8 of angiotensin resulted in antagonists of the receptor [228, 229]. However, while it does generate a $G\alpha_q$ signalling antagonist, these substitutions generate partial agonists of other AT1R-coupled pathways.

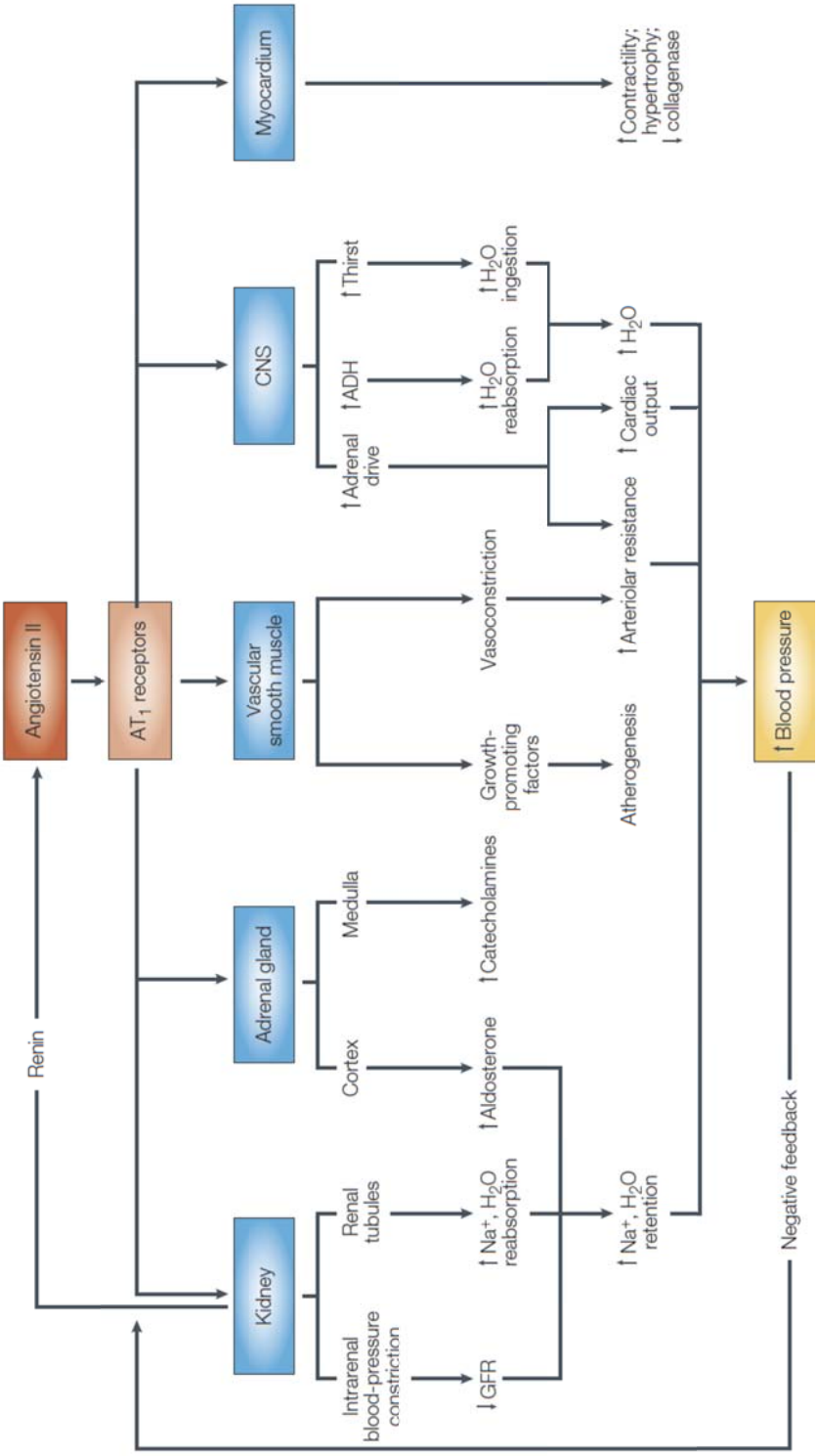

Figure 9. Physiological targets and functions of angiotensin II. AngII acts on the kidney, adrenal gland, myocardium, central nervous system and the vasculature and in concert results in increases in blood pressure [230].

Figure 10. Synthesis of angiotensin II from angiotensinogen and its function at the AT1R [223]. BP = blood pressure

3.3. AT1R signalling

The AT1R is comprised of 359 amino acids and belongs to the rhodopsin-like class of GPCRs (class A) [231]. It is principally coupled to $G\alpha_q$ leading to the activation of PLCβ, which cleaves PIP_2 into IP_3 and DAG [232]. IP_3 acts on its receptor found on the endoplasmic/sarcoplasmic reticulum to release intracellular stores of Ca^{2+} that together with DAG activate PKC [233]. PKC activity has been implicated in the induction of MAPKs including ERK1/2 that proceed to translocate to the nucleus and exert transcriptional effects [234]. The AT1R can be uncoupled from $G\alpha_q$ through the disruption of the conserved DRY motif with point substitutions [15]. The AT1R has also been described to couple to $G\alpha_i$ [235], although this event was reported by only one group, and attempts to demonstrate decreased levels of cAMP downstream of AT1R activation have been unsuccessful. In addition, there was some speculation that $G\alpha_{12/13}$ coupled to the receptor, as activation of Rho kinases and cellular contraction are seen following AT1R activation. However, recent studies have revealed that these effects may be mediated by the Rho exchange factor, Arhgef1 in a $G\alpha_q$-dependent manner [236].

The AT1R has also been described to activate other pathways independent of G protein signalling (some of which depend on βarrestin, including Src, Janus kinase 2 (Jak2) and ERK1/2 [237-239]. AngII-mediated ERK1/2 activation is often subdivided into two phases, an immediate-early phase that is G protein-dependent, and a late-sustained phase that is primarily βarrestin-dependent [240, 241]. Besides their temporal differences, the ERK activated by these two pathways is also believed to be distinguishable spatially with G protein-mediated ERK in the nucleus, while βarrestin-mediated ERK would be constrained to the cytosol by βarrestin itself [213]. The role for ERK1/2 signalling in the cytosol still remains unclear, although multiple cytosolic targets have been identified. AT1R's also activates ERK1/2 by alternative methods including Src, Pyk2 and AT1R-mediated transactivation of receptor tyrosine kinases like the

epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) [242-244].

The AT1R can directly recruit Jak2 through a tetrapeptide motif, YIPP, found in the C-terminal tail of the receptor [245]. Jak2 leads to the activation of signal transducers and activators of transcription (STAT) proteins and promotes their migration to the nucleus where they induce gene transcription.

3.4 AT1R endocytosis

The AT1R undergoes rapid internalization following treatment with AngII in a clathrin-, AP-2- and βarrestin- and dynamin-dependent manner [178]. Originally, there was significant confusion concerning the AT1R with βarrestin dominant negatives failing to block internalization [246] but it is clear now that βarrestin is a major determinant in AT1R endocytosis [247]. In MEF cells, where both βarrestin1/2 are knocked out, over 80% of receptor internalization is lost [112]. Furthermore, previous work from our lab demonstrated that depletion of β_2-adaptin diminished and delayed AT1R endocytosis [179]. However, it is clear that secondary internalization pathways exist for the AT1R and that the degree each pathway is involved may vary between the cellular compartment. While the phosphorylation sites for PKC and the GRKs are largely undetermined, studies have demonstrated that the receptor rapidly becomes phosphorylated following AngII treatment [248, 249].

Following receptor internalization, AT1R colocalizes with βarrestin in endosomes as their interaction is one of high avidity (class B) [250]. While studies examining the compartmentalization of the AT1R following internalization have demonstrated that while the AT1R localizes to Rab5 (early endosomes) and Rab7 (late endosomes) vesicles, the vesicles associated with AT1R recycling are less defined. While Rab11 should be the predominant protein involved, it appears that instead Rab4 may be the principal Rab involved, as it was demonstrated to have a higher binding propensity than Rab11 [251].

However, much remains to be uncovered concerning AT1R recycling and resensitization.

3.5 Physiological relevance of the AT1R

The AT1R's main role involves the regulation of blood pressure through multiple facets [252]. In the vasculature, it acts to increase the peripheral resistance and thus the arterial pressure through vasoconstriction of arterioles [253]. In the adrenal cortex, it acts to stimulate aldosterone release, which acts on the kidneys to increase the retention of both sodium and water [254]. In the central nervous system, the AT1R stimulates the release of vasopressin or antidiuretic hormone from the posterior pituitary, which also causes the kidney to increase fluid retention [255]. In addition, it stimulates the thirst center in the brain and facilitates norepinephrine release while inhibiting its reuptake, thereby enhancing adrenergic action and vasoconstriction [256]. Finally, AT1R activation stimulates both cardiac and vascular hypertrophy [257, 258]. As a result, deregulation of the renin-angiotensin-aldosterone system or AT1R signalling can have profound cardiovascular consequences, and they play a significant role in the pathophysiology of hypertension and congestive heart failure.

4. Biased or ligand-directed signalling

Classically, an agonist acting at a receptor was thought to equallty activate all coupled signalling pathways. Antagonists were characterized as compounds, which competitively or non-competitive block the agonist effect. However, only the primary pathway coupled to the receptor was usually tested, as a result of technical limitations, the concept of ligand-directed signalling went unnoticed. As new assays were developed, it became apparent for various receptor systems that ligands could selectively activate or inhibit receptor pathways independent of others. Furthermore, it was realized that many GPCR antagonists actually activated βarrestin-dependent signalling [259]. The concept focuses on the differential conformations imposed by various ligands on the same receptor can induce selective coupling [260].

While the realization of selective signalling occurred approximately 10 years ago [139], numerous receptor systems have been demonstrated to display this bias, both *in vitro* and *in vivo.* One of the most characterized receptor in terms of ligand bias is the β_2-adrenergic receptor as a result of the numerous ligands available [261]. For instance, 3 ligands of the receptor, isoproterenol, bucindolol and propranolol, a full, partial and inverse agonist of the adenylyl cyclase pathway, respectively, all activate MAPK [262]. Furthermore, it was demonstrated that while isoproterenol caused MAPK activation through both $G\alpha_i$ and non-G protein-dependent, bucindolol and propranolol only used G protein-independent methods. Further complicating the situation were neutral antagonists to the MAPK pathway, while maintaining some coupling to adenylyl cyclase [263]. The AT1R also represents a well-characterized receptor for ligand biased signalling as a result of the angiotensin analog [Sar^1,Ile^4,Ile^8]-AngII (SII). SII is one of the first ligands where the biased signalling potential was realized, as it possesses no activity at the $G\alpha_Q$ activation and IP_3 formation, while being a partial agonist at βarrestin recruitment and MAPK activation [264]. Numerous studies have confirmed the bias at the AT1R and as discussed below, biased ligands are being explored for their potential therapeutic benefit [225, 265]. Figure 10 gives a pictorial representation of how ligand-biased signalling may occur.

The mechanism of ligand biasing signalling occurs is still a matter of debate, although the most likely explanation involves the differential receptor conformation imparted by ligand binding. Some studies have tried to demonstrate this largely through fluorescent approaches and

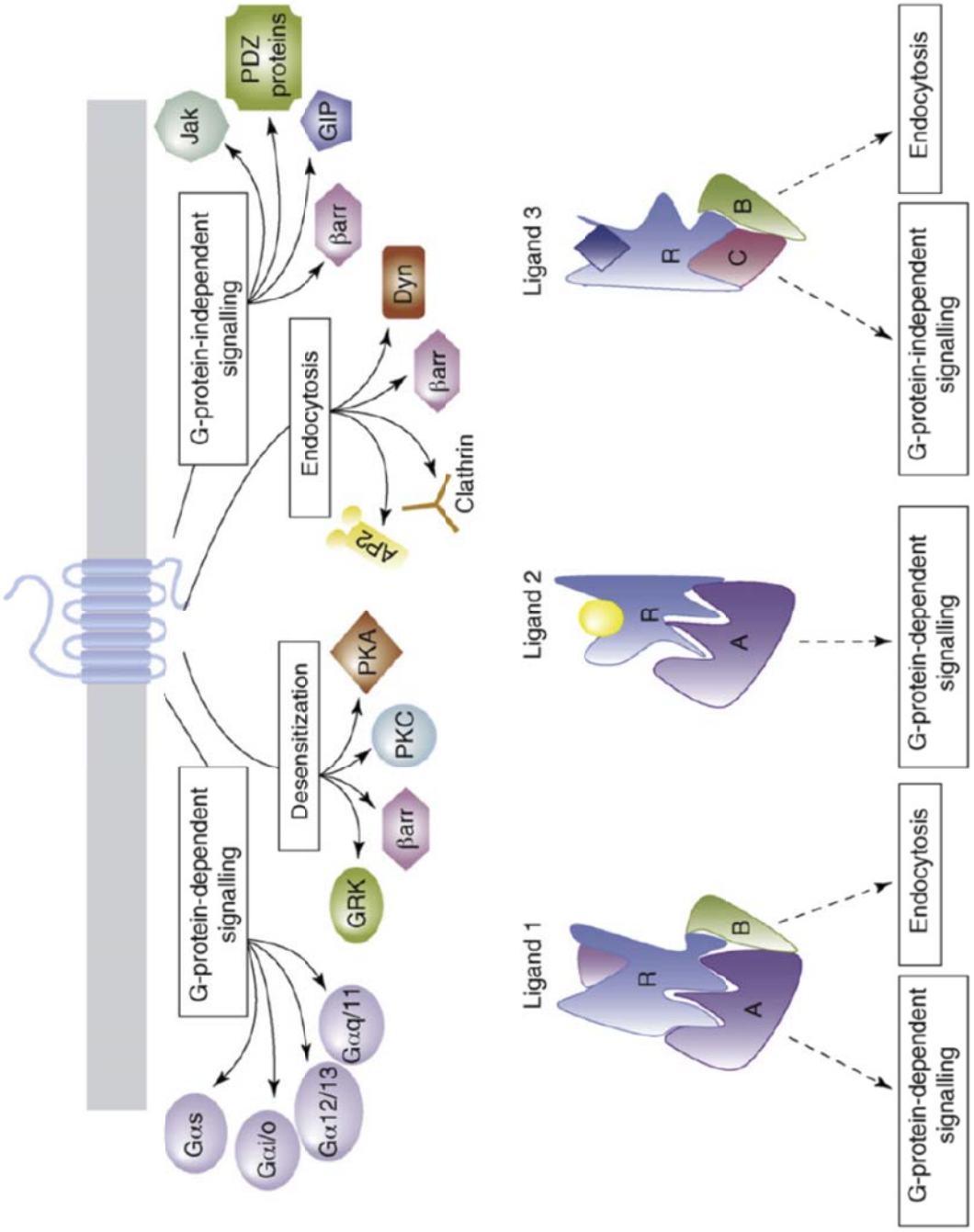

Figure 11. Ligand biased signalling allows for selective activation of downstream signalling pathways-dependent on the receptor conformation imparted by the ligand bound [266].

reveal that the fluorescent signature of the receptor changes based on the ligand. Using a fluorescently-labelled β_2-adrenergic receptor, they observed different fluorescence patterns depending on the orthosteric agonist used [267]. In fact, this phenotype had been observed long before the concept of ligand biased signalling even really existed, where the *Kobilka* lab saw structural differences in the purified receptor depending on co-precipitated [268]. More recent studies revealed similar effects with both the α_2-adrenergic and adenosine A_2 receptor suggesting that ligand-directed signalling may be the result of conformational bias [269, 270]. Aside from the actual receptor conformation, other factors can also affect how associated proteins interact with the receptor, including GRK-mediated phosphorylation. A recent study revealed that signalling through βarrestin varied dependent on the level and sites of GRK phosphorylation [102]. This study suggests that not only can the receptor's conformational structure affect signalling but post-translational modifications can also produce a significant effect.

Finally, there is much excitement over ligand-directed signalling for potential new therapeutics. One example involves the parathyroid hormone receptor (PTH1R), where a selective βarrestin-signalling ligand, (D-Trp^{12},Tyr^{34})-PTH(7–34) demonstrated that biased activation of βarrestin was potentially of therapeutic benefit because it only led to trabecular bone formation, as opposed to general PTH1R agonists that cause formation and resorption of bone [271]. Perhaps the most advanced example involves the AT1R, which currently has a biased ligand, an AngII analog, at the phase II clinical trials. The peptide, which has been characterized by the company Trevena and named TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH), has been demonstrated to have therapeutic benefits in excess of classical receptor ligands at the AT1R, ARBs. The initial study demonstrated that TRV120027 was capable of reducing the pathological actions of AngII to increase BP, while maintaining its beneficial effects on cardiac performance [225]. They established that this was a result of selective action of

AT1R-mediated βarrestin signalling without the activation of concomitant G protein signalling. Phase I trials demonstrated the *in vitro* and *in vivo* effects they saw in animals were reproducible in humans, and thus the peptide is being evaluated for safety in phase II trials. Unfortunately, peptide drugs are not ideal, as they do not allow for oral administration due to their metabolism by peptidases in the gastrointestinal tract. While this peptide is still effective if given intravenously, it is likely that the development of peptidomimetics is underway, as they are orally available.

5. Single chain antibodies

Single chain antibodies (also known as single chain variable fragment – scFv) are constructed from the variable regions of both the heavy and light chain of immunoglobulins that are connected by a 10-25 amino acid linker composed primarily of glycine, serine and threonine for flexibility and solubility [272]. Despite the removal of the constant regions of the immunoglobulin, they maintain specificity for the original antigen. The loss of the constant region also removed common binding sites including those for protein G. As such, purification of single chain antibodies requires protein L, as it interacts with the variable region of K light chains. Despite the loss of its binding site, scFvs still bind to protein A through an unknown region. A considerable benefit of single chain antibodies involves the ability to generate them directly from subcloned heavy and lights chains derived from a hybridoma [273]. Conventionally, scFvs are produced in bacteria, compared to monoclonal antibodies (mAbs) that are synthesized in mammalian cultures. However, as we actually have the coding sequence to generate these antibodies, as opposed to mAbs where we only have cells that express the protein, this allows for the potential targeted expression of within cells. In doing so, this overcomes a major hurdle encountered with mAbs that target intracellular epitopes, as it is a difficult and often disruptive process to the cell to facilitate their entry.

An early study using scFvs demonstrated that transfection of an antibody directed against a protein involved in cervical cancer progression, bound to the protein leading to its downregulation andinhibition of cell growth, highlighting a potential therapeutic role for this scFv [274]. Clinically, a drug called pexelizumab is actually a scFv that binds to protein 5 of the complement system. It prevents the inflammatory actions of the complement system, and is indicated in patients post coronary artery bypass grafting, and angioplasty among other forms of cardiac surgery [275]. At a more mechanistic level, a scFv directed against ErbB2 (a receptor whose signalling roles were unclear at the time), caused its intracellular retention. ErbB2 requires the dimerization with other members of the EGFR family, therefore, the loss in signalling mediated by this scFv following stimulation with EGF or Neu differentiation factor could be attributed to this receptor [276]. As such, while these antibodies could play a significant role therapeutically, they may also serve as effective tools to dissect signalling pathways and protein interactors.

Attempts to increase the affinity of scFvs for their substrates, whether intracellular or extracellular, have actually been quite fruitful. This affinity increase is the result of antibody multimerization. Dimeric or divalent scFvs are generated by including two variables regions from the light and heavy chain in a single construct [277]. Alternatively, one can use extremely short linkers, about 5 amino acids in length, which prevent the interaction between the two variable fragments. Instead, it is forced to interact with the light or heavy variable region of another scFv, generating, diabodies [278]. It was demonstrated that the diabody may have up to 40 fold greater affinity for the antigen compared to the monomeric scFv. Further increases in the affinity are possible through the tribodiy or tetrabody generation with affinity increasing with each successive addition [279]. This affinity increase is important, as removal of the constant domains of the immunoglobulin diminishes the potency for an antigen.

6. Rationale

G protein-coupled receptors play significant physiological roles in many of functions of our everyday lives. The signalling downstream of these receptors is subject to numerous forms of regulation involving desensitization, internalization, and resensitization. However, each of these processes is not static and independent of each other and, therefore, they can modulate one another. For instance, modulation of receptor desensitization through alterations in phosphorylation of the GPCR will also impact receptor internalization. Likewise, if we were to inhibit the endocytic process, this would lead to improper resensitization of many GPCRs, leading to aberrant signalling.

The mechanism of endocytosis leading to GPCR internalization varies dependent on the receptor, however, the most predominantly used pathway involves clathrin. Clathrin-mediated endocytosis for GPCRs involves the initial recruitment of βarrestin to the receptor, which has been phosphorylated on conserved serine and threonine residues found in the third intracellular loop and the C-terminal tail of the receptor. This phosphorylation creates the high affinity binding site for βarrestin recruitment; loss of these sites frequently leads to the absence of internalization. Following βarrestin binding, receptors are targeted towards specific regions on the plasma membrane called clathrin coated pits (CCPs), which are regions of concentrated internalization machinery. While βarrestin can interact directly with clathrin, the recruitment of the clathrin adaptor protein, AP-2, through its β-subunit ($\beta_2$adaptin), usually precedes clathrin binding, and is a significant driving force in targeting the cargo, (*i.e.* the GPCR) to these CCPs. In order for βarrestin to bind to clathrin, it must dissociate from the $\beta_2$adaptin, as the binding site on βarrestin for AP-2 and clathrin are in close proximity. Work from our lab found that for efficient dissociation of the complex, phosphorylation from the non-receptor tyrosine kinase, Src, was required. This work was performed in our lab by a previous Doctoral student, Dr.

Delphine Fessart, a post-doctoral fellow, Dr. May Simaan and by myself, where we established through numerous techniques that phosphorylation of $\beta_2$adaptin was involved [179]. Using *in vitro* techniques and mutagenesis, the putative phosphorylation site was located, and my work demonstrated that substitution of this tyrosine with phenylalanine slowed the dissociation and thus stabilized their interaction. This manuscript is not included in the thesis, as it was partially entered into the thesis of Dr. Delphine Fessart, although my work on the manuscript was performed following the submission of her thesis. Once βarrestin is free from AP-2, it is able to bind to clathrin, and upon the vesicle reaching critical mass, dynamin becomes activated by its conversion of GTP to GDP, leading to pit scission from the plasma membrane and the formation of a clathrin coated vesicle.

While much research has focused on how internalization regulates signalling, the objective of this thesis largely deals with how signalling can regulate internalization. As AP-2 and βarrestin are the main adaptors involved in clathrin-mediated endocytosis, the manuscripts presented here examine how $\beta_2$adaptin of AP-2 and βarrestin can be regulated or modified by signalling events to modify receptor trafficking or endocytosis that potentially can lead to altered signalling downstream of these endocytic adaptors.

Using the AT1R as a model receptor, the main goals of this thesis were three fold; 1) determine if $\beta_2$adaptin was actually phosphorylated in cells upon receptor activation, 2) establish a functional role for $\beta_2$adaptin phosphorylation in regards to receptor trafficking or signalling and 3) determine how multiple ligands at the same receptor that induce differential signalling paradigms can regulate βarrestin's functions as a GPCR adaptor. Together, we hope to reveal how signalling events can regulate the function of endocytic adaptors.

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Connecting Text

The following section (**Chapter 2**) entitled, "c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway" demonstrated in living cells that $\beta_2$adaptin of the AP-2 subunit becomes tyrosine phosphorylated on a specific residue in response to receptor activation. Our objective was to determine whether this event occurred, the localization of the event and the biochemical players involved. While our previous work had established a potential phosphorylation event through mutagenesis work, it did not demonstrate that it was indeed phosphorylation of the residue which was necessary. Furthermore, the proteins required and the ability of extracellular stimuli to induce the stimuli were unknown. Taken together our main goals were to ascertain:

1) Whether the polyclonal antibody we generated was capable of specifically recognizing phosphorylated Y737 $\beta_2$adaptin,

2) Whether $\beta_2$adaptin phosphorylated on tyrosine 737 in cells occurs following overexpression of tyrosine kinases or receptor activation,

3) Which receptors are capable of eliciting this phosphorylation event, if any,

4) Which proteins play an essential for the phosphorylation event to occur, beyond the kinase?

Chapter 2

c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway

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Abstract

Clathrin-mediated endocytosis is a complex process regulated at many different levels. We showed previously that activation of the angiotensin type 1 receptor (AT1R), which belongs to the G protein-coupled receptor (GPCR) family, leads to c-Src-dependent tyrosine phosphorylation of β2-adaptin, a subunit of the clathrin adaptor AP-2. The phosphorylation of β2-adaptin on tyrosine residue 737 (Y737) negatively regulates its interaction with βarrestin, another important clathrin adaptor for GPCR internalization. Here we sought to determine whether AP-2 phosphorylation represents a general mechanism for different receptors internalizing through the clathrin pathway. Using a specifically designed antibody against the phosphorylated form of Y737 on β2-adaptin, we demonstrate that this residue is phosphorylated by AT1R in different cell types like HEK293, COS-7 and vascular smooth muscle cells. Using RNA interference approaches, we reveal that this agonist-mediated event is both βarrestin- and c-Src-dependent, and that it occurs at the plasma membrane in clathrin-coated vesicles (CCVs). We further show that this is not only a common event employed by other GPCRs like the β2-adrenergic, vasopressin V2, bradykinin type 2, platelet-activating factor and endothelin A receptors but that the epidermal growth factor receptor is capable of eliciting the phosphorylation of AP-2 in CCVs. Our results imply that tyrosine phosphorylation of Y737 on β2-adaptin is a common regulatory mechanism employed by different receptors undergoing clathrin-dependent endocytosis, and suggest a wider function for this event than originally anticipated.

Introduction

Seven transmembrane receptors (also referred to as G protein-coupled receptors [GPCRs], when they are functionally activating heterotrimeric G proteins) are integral membrane proteins responsible for controlling an array of physiological responses such as phototransduction, olfaction, vascular tone, cardiac output and pain. Cellular responses mediated by the activation of these receptors are modulated by processes that regulate either positively or negatively the receptors' coupling with their downstream effectors. Processes that rapidly abrogate cell signaling include desensitization, where receptors become refractory to repeated stimuli, and internalization (also referred to as endocytosis), which involves the removal of receptors from the plasma membrane [1, 2].

Internalization of GPCRs can lead to continued intracellular signaling of the receptor, the reestablishment of the cellular response through recycling of the receptor to the plasma membrane, or its prevention by targeting the internalized receptor for degradation to lysosomes. One of the most used and best-characterized internalization routes is the clathrin pathway. Central to the clathrin-dependent internalization of GPCRs, like the β2-adrenergic receptor (β2AR) and the angiotensin type 1 receptor (AT1R), is βarrestin. βarrestin acts as an adaptor to target desensitized receptors to clathrin-coated vesicles (CCVs) through its interaction with two major coat constituents: clathrin and the heterotetrameric AP-2 complex (specifically through the β-subunit, referred hereafter as β2-adaptin) [3-6].

Mechanisms regulating the clathrin-dependent internalization of receptors are still not fully understood. Accumulating evidence, however, suggests that recruitment of kinases and the phosphorylation of proteins of the coat are important to this process [7-14]. For instance, βarrestin has been shown to recruit c-Src to phosphorylate dynamin, a GTPase involved in the

scission of CCVs, and to regulate β2AR internalization [7, 13, 14]. Phosphorylation of clathrin and dynamin by c-Src is also involved in the internalization of the epidermal growth factor receptor (EGFR) [11]. The different subunits of the AP-2 complex are also a target for phosphorylation [8-10, 12]. In particular, β2-adaptin has been shown to be tyrosine phosphorylated on its N-terminal domain (*i.e.* on tyrosine 6, Y6) following EGFR activation [12]. The mechanism and role for the EGFR-mediated β2-adaptin phosphorylation remain unknown. We recently revealed that β2-adaptin was phosphorylated on its C-terminal domain after agonist stimulation of the AT1R [9]. We show that this phosphorylation event is mediated by c-Src and that tyrosine 737 (Y737) is an important residue regulating the binding of AP-2 to βarrestin [9, 15]. Although we have identified this regulatory site in β2-adaptin through mutagenesis, it remains unknown whether Y737 is a target *in vivo* and what signaling events regulate the phosphorylation of AP-2 in cells.

To gain a better understanding of the underlying mechanisms involved in the AT1R-mediated phosphorylation of AP-2, and to determine whether Y737 in β2-adaptin represents a general target in cells for different receptors internalizing through the clathrin pathway, we have developed an antibody to that specific phosphosite. Here we present evidence that c-Src phosphorylates AP-2 on Y737 of β2-adaptin in different cell types; this mode of regulation is not limited to the AT1R, but is also seen with other GPCRs and receptor tyrosine kinases like the EGFR.

Results

AT1R activation induces phosphorylation of β2-adaptin on tyrosine 737

We have previously shown that AT1R activation in VSMCs, COS-7 and HEK293 cells lead to an increase in total tyrosine phosphorylation of β2-adaptin [9]. Furthermore, mutagenesis data revealed that Y737 of β2-adaptin is a putative c-Src phosphorylation target and is an important regulator of the βarrestin/AP-2 complex. To determine if this site is a phosphorylation target in cells, we set out to develop an antibody specifically directed against phosphorylated Y737 of β2-adaptin. We examined if the antibody (herein referred to as AP-4213) could specifically recognize phosphorylated β2-adaptin. We initially attempted to detect the phosphorylation of AP-2 using total cell lysates without great success. Therefore, we decided to enrich AP-2 through immunoprecipitation before detecting its phosphorylation status. We first verified that AP-4213 recognizes β2-adaptin phosphorylated at Y737 using cells transiently expressing Flag-β2-adaptin-YFP in the presence of c-Src (Fig. 1A). When AP-2 complexes are immunoprecipitated (using anti-Flag or anti-AP-1/2 antibodies) we observe an increase in the amount of endogenous and transfected β2-adaptin that is immunoreactive to AP-4213 in c-Src-overexpressing cells. Similarly, when AP-4213 was used to immunoprecipitate either transfected or endogenous β2-adaptin, we see an increase in immunoreactive β2-adaptin in the presence of c-Src. We consistently observe cleaner immunoreactive signals on blots when using the AP-4213 antibody in immunoprecipitations. Thus, we decided to continue using this antibody to immunoprecipitate phosphorylated AP-2. To further demonstrate that the phosphorylation observed is specific to Y737, cells were left untransfected or transfected with c-Src and either Flag-β2-adaptin or Flag-β2-adaptin-Y737F, before subjecting cell lysates to immunoprecipitation with AP-4213. Although the antibody is able to immunoprecipitate phosphorylated β2-adaptin in both wild-type transfected and control cells

expressing c-Src, no β2-adaptin is immunoprecipitated in cells transfected with Flag-β2-adaptin-Y737F (Fig. 1B), thereby demonstrating that the antibody specifically recognizes phosphotyrosine 737. Given this finding, we then assessed whether agonist activation of the AT1R leads to the phosphorylation of β2-adaptin on this site. Using VSMCs expressing endogenous receptors, and AT1R-transfected COS-7 and HEK293 cells, we observe that in response to Ang II, endogenous β2-adaptin becomes phosphorylated at Y737 in a time-dependent manner (Fig. 2A, B and C, respectively). Densitometry analysis reveals that the phosphorylation reaches a maximum after 5 minutes of Ang II treatment and is maintained after 15 minutes of agonist stimulation (Fig. 2A/B/C, graph).

Phosphorylation of tyrosine 737 on β2-adaptin is not unique to AT1R stimulation

Given our finding that phosphorylation of β2-adaptin at Y737 played a role in the clathrin-mediated internalization of AT1R, we hypothesized that this phosphorylation event may be common to other GPCRs. We tested whether the β2-adrenergic receptor (β2AR), the bradykinin type 2 receptor (B2R), the platelet-activating factor receptor (PAFR) or the vasopressin V2 receptor (V2R), could phosphorylate tyrosine 737 on β2-adaptin following receptor activation [16, 17]. As seen in fig. 3A, all four receptors induce agonist-driven Y737 phosphorylation of β2-adaptin. To demonstrate that this phosphorylation event is a feature of GPCRs internalizing through the clathrin pathway, we used the endothelin type A and B receptors (ETAR and ETBR). These receptors can be activated by the same ligand but have been suggested to internalize differentially through either the clathrin- or caveolae-mediated pathways, respectively [18-21]. Results show that although both endothelin receptors are able to activate extracellular signal-regulated kinase 1/2 (ERK1/2) upon endothelin treatment, only ETAR induces phosphorylation of β2-adaptin (Fig. 3B). Finally, we tested whether a receptor tyrosine kinase (RTK), the epidermal growth factor receptor

(EGFR), that is known to internalize through the clathrin pathway, can also elicit phosphorylation of β2-adaptin [22]. Cells transfected with EGFR were treated with epidermal growth factor (EGF) and the phosphorylation status of Y737 was examined. Results show that similarly to other GPCRs tested, stimulation of EGFR induces the phosphorylation of β2-adaptin on Y737 (Fig. 3C).

Phosphorylation of tyrosine 737 occurs at the plasma membrane in clathrin-coated vesicles

We next sought to determine where in the cell Y737 phosphorylation of β2-adaptin was taking place. To visualize the localization of this event, we performed immunofluorescence studies using AP-4213. First, to validate the specificity of the signal, cells were transfected with AT1R and a shRNA against β2-adaptin. Transfection of shRNA results in a total protein knockdown (Fig. 4A). The shRNA vector also expresses GFP, which we use to correlate the knockdown efficiency to the level of β2-adaptin phosphorylation. Cells expressing high levels of GFP, gauged by fluorescence intensity, should have substantial β2-adaptin knockdown, whereas cells expressing lower levels of GFP should have less knockdown of β2-adaptin. As seen in fig. 4B, the cell expressing a large amount of GFP has minimal phosphorylated β2-adaptin staining at the plasma membrane following AT1R activation. On the other hand, the cell with a haze of GFP expression shows strong AP-4213 staining. To further localize this phosphorylation, immunofluorescence was performed on cells expressing either AT1R or EGFR and β2-adaptin-YFP. Both AT1R and EGFR activation, as seen in fig. 4C, result in strong staining of AP-4213 at or near the plasma membrane with signals colocalizing with β2-adaptin-YFP (Fig. 4C insets for AT1R, and not shown for EGFR). Since β2-adaptin-YFP colocalized with AP-4213 staining, our results indicate that phosphorylation of AP-2 occurs mainly in CCVs.

AT1R mediated β2-adaptin Y737 phosphorylation is dependent on βarrestin

Our previous work suggested that agonist-mediated tyrosine phosphorylation of β2-adaptin required βarrestin [9]. We first wanted to demonstrate that G protein involvement was not required for β2-adaptin Y737 phosphorylation. To do this, we used a mutant of AT1R (Y302A) that we have previously shown to be functionally uncoupled from its G protein but still undergoes internalization [23]. As seen in fig. 5A, this AT1R mutant is still capable of eliciting β2-adaptin phosphorylation on Y737, and ERK1/2 activation. Additional evidence of a G protein independent process is revealed through the use of an Ang II analog, $Sar^{1}Ile^{4}Ile^{8}$ (SII). This analog has been shown to induce βarrestin recruitment to AT1R and its internalization without inducing receptor-mediated G protein signaling [24]. As seen in fig. 5A, treatment with SII results in both the phosphorylation of β2-adaptin and ERK1/2 activation comparable to Ang II-mediated stimulation. However, the non-peptide AT1R antagonist (L158,809) does not elicit either phosphorylation of β2-adaptin, or ERK1/2 activation. These results imply that the phosphorylation of Y737 is a G protein-independent event, which we suspect to be dependent on the signaling function of βarrestin. To validate this hypothesis, we took advantage of a HEK293 stable cell line expressing doxycycline-inducible shRNA against both forms of βarrestin. As seen in fig. 5B, doxycycline treatment of cells results in the knockdown of both βarrestin1 and βarrestin2. Under these conditions, time-dependent phosphorylation of β2-adaptin Y737 was substantially decreased as compared to control cells.

β2-adaptin Y737 phosphorylation by AT1R and EGFR is c-Src dependent

Based on our previous results implicating c-Src in the total β2-adaptin phosphorylation, we next verified the involvement of Src family kinases in β2-adaptin Y737 phosphorylation. The non-selective Src kinase family inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), is able to significantly diminish Y737 phosphorylation of β2-adaptin in response to either

AT1R or EGFR activation (Fig. 6A). To next demonstrate the specific involvement of c-Src in the phosphorylation of β2-adaptin, we used a siRNA knockdown approach. As seen in fig. 6B, siRNA against c-Src reduces its endogenous expression in cells by at least 50%. Loss of the c-Src kinase leads to a decrease in Ang II-mediated phosphorylation of β2-adaptin. These results reveal that β2-adaptin Y737 phosphorylation following AT1R and EGFR is mediated by c-Src kinase activity.

Discussion

Our study provides further evidence that Y737 of the β-subunit of AP-2 is a c-Src target in cells, which is regulated in CCVs during the internalization of receptors. Using an antibody against the phosphorylated form of Y737, we show that AP-2 phosphorylation occurs at or near the plasma membrane in CCVs. We also demonstrate for AT1R that this event requires both βarrestin and c-Src, and that other GPCRs known to internalize through the clathrin pathway are capable of promoting the tyrosine phosphorylation of AP-2. Finally, we reveal that EGFR activation elicits the c-Src-dependent tyrosine phosphorylation of AP-2 in CCVs, which suggests that Y737 on β2-adaptin represents an important regulatory site for different classes of membrane receptors internalizing through the clathrin pathway.

Clathrin-mediated internalization of receptors requires the coordinate action of many different adaptors and signaling effectors. Our previous findings on the AT1R internalization suggested that the recruitment of the non-receptor tyrosine c-Src through βarrestin, led to the phosphorylation of AP-2 in CCVs [9, 15]. Moreover, we showed using a mutagenesis approach that Y737 on β2-adaptin is an important regulatory site, and that its potential c-Src-mediated phosphorylation is involved in the dissociation of βarrestin/AP-2 complexes during AT1R internalization. Here using a selective antibody against Y737, we demonstrate that this site is indeed a target in cells and that it is phosphorylated at or near the plasma membrane in CCVs. The localization of AP-2 phosphorylation supports our earlier results, as functionally, Y737 phosphorylation is important for the disruption of the βarrestin/AP-2 complex, which logically would occur in CCVs during receptor internalization.

Our findings provide further evidence that β2-adaptin phosphorylation requires both βarrestin and c-Src. Knockdown of either protein using a RNA interference strategy leads to a loss in agonist-mediated Y737 phosphorylation of

β2-adaptin following AT1R activation. Moreover, Ang II-mediated activation of an AT1R mutant that is uncoupled from its G protein, or stimulation of AT1R with a ligand that does not promote its functional coupling to Gq but still promotes the binding of βarrestin and receptor internalization, are still able to induce AP-2 phosphorylation [23, 24]. Together these results support the role of βarrestin as a signaling scaffold in the regulation of receptor internalization through the clathrin pathway.

We show that other GPCRs like the β2AR, B2R, V2R, PAFR and ETAR, which are known to recruit βarrestin to agonist-activated receptors, are also able to induce the phosphorylation of AP-2. At present, we can only speculate whether this phosphorylation event also requires the βarrestin-dependent recruitment of c-Src to these different GPCRs. Indeed, binding of βarrestin to activated receptors appears insufficient to induce the phosphorylation of AP-2. Agonist activation of the ETAR, but not the ETBR, induced the phosphorylation of AP-2, despite the fact that both receptors were equally able to promote the agonist-mediated recruitment of βarrestin to receptors [18, 19]. Moreover, we observed that activation of EGFR induces the phosphorylation of β2-adaptin on Y737. Although another receptor tyrosine kinase, the insulin-like growth factor receptor (IGF1R), has been shown to interact with βarrestin, as far as we know, EGFR does not [25, 26]. We demonstrate that EGFR-mediated AP-2 phosphorylation is dependent on the activity of Src kinases, indicating common endocytic regulators between AT1R and EGFR. Moreover, results obtained with ETAR and EGFR suggest that different signaling mechanisms, independent of βarrestin, can be involved in regulating the phosphorylation of β2-adaptin on Y737.

AP-2 phosphorylation in the context of clathrin-dependent internalization of GPCRs was investigated through the use of different receptors that were reported to utilize that route of endocytosis. The β2AR, B2R, AT1R, PAFR and

V2R have all been described to internalize through the clathrin pathway [6, 21, 27-31]. The findings presented here on the tyrosine phosphorylation of β2-adaptin are consistent with our previous results showing that the activation of the β2AR, B2R, AT1R and V2R promotes the formation of βarrestin/AP-2 complexes [9, 19]. On the other hand, the internalization pathway used by the ETAR and ETBR is still subject to controversy. Previous studies reported conflicting results highlighting the fact that both receptors could undergo clathrin- and/or caveolin-dependent endocytosis using different cell lines and assays [20, 21, 32, 33]. Our results reveal that stimulation of ETAR induces tyrosine phosphorylation of β2-adaptin, consistent with its internalization through the clathrin pathway; whereas the lack of ETBR-mediated AP-2 phosphorylation suggests that this receptor uses an alternative mechanism and/or route of endocytosis.

EGFR is also known to internalize through the clathrin pathway, and has been shown to induce the tyrosine phosphorylation of β2-adaptin in its N-terminal domain (Y6) [12]. However, it remains to be determined whether this site is indeed phosphorylated in cells and what is the underlying mechanism regulating this specific event. Here we show that stimulation of EGFR leads to phosphorylation of β2-adaptin on Y737 in its C-terminal domain, which was dependent on the activity of Src family kinases. Interestingly, two recent studies using a large phosphoproteomic survey have identified this site as being phosphorylated in Src-transformed cell lines and EGFR-dependent tumors [34, 35], supporting our contention that Y737 of β2-adaptin is a phosphorylation target *in vivo*.

We recently advanced a functional role for the phosphorylation of β2-adaptin on Y737 during the clathrin-mediated internalization of AT1R. We showed using a β2-adaptin mutant that Y737 phosphorylation is necessary for the dissociation of AP-2 complexes from βarrestin in CCVs [9]. Thus, AP-2

phosphorylation induced by β2AR, B2R, and V2R receptors may also play a role in the dissociation of βarrestin/AP-2 complexes during the internalization of receptors [19]. The PAFR is also capable of recruiting βarrestin and to internalize through the clathrin pathway [36]. However, it remains to be determined whether PAFR can promote the formation of βarrestin/AP-2 complexes and whether AP-2 phosphorylation can regulate the disassembly of these complexes. As for the ETAR, although this receptor could recruit βarrestin, it did not promote a detectable interaction between βarrestin and AP-2 following receptor activation [19], suggesting a different functional role of Y737 phosphorylation in the internalization of this receptor. The role of β2-adaptin Y737 phosphorylation in the regulation of EGFR endocytosis is also unknown. Since β2-adaptin interacts with other accessory proteins of the coat (AP180, eps15, epsin, amphiphysin, and clathrin) [37, 38], it is possible that phosphorylation of AP-2 may regulate the binding of β2-adaptin with these other endocytic proteins. AP-2 is also known to directly interact with some GPCRs and EGFR, thus the phosphorylation of AP-2 could regulate its own binding to these receptors [39-41]. These possibilities will require further investigation.

Conclusion

We show that Y737 on β2-adaptin is a phosphorylation target in cells for many different GPCRs and for a RTK, the EGFR. We demonstrate that AT1R- and EGFR-mediated AP-2 phosphorylation occurs in CCVs at or near the plasma membrane and that Src kinase activity is essential for this process. Our data further suggests that phosphorylation of β2-adaptin Y737 plays an important and more diverse role than expected in the internalization of different classes of receptors.

Materials and Methods

Materials

Angiotensin II, bradykinin, isoproterenol, vasopressin and L158,809 were from Sigma Chemical Co. The antibody against β-adaptin is from BD Transduction Laboratories. The anti-FLAG and AP-1/2 antibodies as well as the anti-mouse and anti-rabbit HRP conjugated secondary antibodies are from Sigma-Aldrich. Protein G agarose is from Roche. Phospho-p42/p44 (pERK1/2) and p42/p44 (ERK1/2) antibodies are from Cell Signalling. GD11 (anti-src antibody) was purchased from Millipore. Alexa fluor 568 anti-rabbit secondary antibodies are from Molecular Probes. siRNA for c-Src and a non-silencing siRNA are from Qiagen. PP2 is from Calbiochem and DMSO is from Bioshop. β2-adaptin shRNA GFP was purchased from Open Biosystems. Antibodies AP-4213 (anti-phospho Y737 β2-adaptin) and 3978 (anti-βarrestin) were generated at McGill University's animal facility. Briefly for 3978, rabbits were immunized with a purified GST-fusion of the C-terminal domain of βarrestin2 (residues 377-408) to create a polyclonal antibody against the C-terminal domain of βarrestins.

Antibody Generation

In order to generate an antibody against the phospho-tyrosine 737, the peptide GHIY(P)MEMNC was synthesized using conventional solid phase synthesis (Institute of Pharmacology, Université de Sherbrooke) and coupled to Keyhole Limpet Haemocyanin (KLH) through the free cysteine residue using a SulfoLink Immobilization kit (Pierce Biotechnology). The conjugated peptide was injected into 2 different rabbits and immunized following standard procedures with approximately 200 µg of conjugated peptide (McGill University's animal facility). The immunized rabbits were tagged 4212 and 4213. All experiments herein used crude sera from 4213 (AP-4213) with antibody selection being based on the

potency and specific reactivity towards the phospho-peptide compared to the non-phospho-peptide using serial dilution of each peptide in a slot blot assay.

Plasmids and constructs

HA-AT1R, HA-β2AR, HA-B2R, EGFR, wild-type c-Src, Flag-β2-adaptin, siRNA insensitive Flag-β2-adaptin-YFP and Flag-β2-adaptin-Y737F-YFP mutants were described elsewhere [6, 9, 15, 16, 19, 23, 42]. HA-ETAR and HA-ETBR were kindly provided by Dr. Audrey Claing (University of Montreal, Quebec, Canada). Myc-PAFR was provided by the CTiGAR (Canadian Institutes of Health Research Team grant in G protein-coupled receptors Allosteric Regulation, University of Montreal, Quebec, Canada). Flag-β2-adaptin-Y737F was generated by PCR and cloned into Flag-β2-adaptin using BspE1 and KpnI [15]. All constructs were analyzed by DNA sequencing (Sequencing Service, Genome Quebec Innovation Centre, McGill University, Quebec, Canada).

Cell culture and transfection

Human Embryonic Kidney (HEK293) cells were grown in MEM (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and gentamicin (100 μg/ml, Gibco). Cells seeded in a 100-mm dish, at a density of $1.2x10^6$ cells per dish, were transfected using a calcium phosphate co-precipitation method as previously described [15]. COS-7 cells were grown in DMEM (Hyclone) supplemented with 10% heat-inactivated FBS and gentamicin. Transfection of COS-7 cells seeded in 100-mm dishes (at a density of $1.5x10^6$ cells per dish) was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations using a 1:2 ratio of DNA:Lipofectamine in Opti-MEM (Gibco). All experiments were performed 48 hours post-transfection. Vascular smooth muscle cells (VSMCs) were grown in low-glucose DMEM (Gibco) with 10% heat-inactivated fetal calf serum (Gibco) and gentamicin. All cells were serum-starved overnight before performing the experiments. HEK293 cells stably expressing HA-AT1R were a generous gift from Marc G. Caron (Duke University,

North Carolina). A HEK293 cell line carrying TET-inducible shRNA targeting both βarrestin isoforms (CGTCCACGTCACCAACAAC) was generated by co-transfection of pcDNA5/FRT/TO with pOG44 encoding Flp-recombinase (1:10 pcDNA5:pOG44) using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA). After 2 days, recombinant cells were selected in 50 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) and 5 μg/ml blasticidin. These cells were grown in DMEM (Gibco) with 10% heat-inactivated tetracycline-free FBS (Clontech) and gentamicin. Cells were seeded in 100-mm dishes (at a density of $1.5x10^6$ cells) and transfected the following day using a conventional calcium phosphate co-precipitation method. The following day cells were either left untreated or treated with 1 μg/ml doxycycline. Cells were harvested 72 hours after addition of doxycycline for analysis.

Immunoprecipitation and western blot experiments

For phosphoprotein detection experiments, COS-7, HEK293 or VSM cells were serum-starved overnight, and the next day incubated for 30 minutes at 37°C in their respective media, buffered with 20 mM HEPES pH 7.4. For experiments involving PP2, cells were incubated with either DMSO or PP2 (5 μM) for 30 minutes prior to agonist stimulation. Cells were then treated with Angiotensin II (Ang II) (1 μM) for the indicated period of time. Stimulation was stopped on ice, washed with ice-cold PBS, and cells were solubilized in TGH buffer (50 mM HEPES pH 7.4, 1% Triton X-100 (v/v), 10% glycerol (v/v), 50 mM NaCl, 5 mM EDTA) containing protease and phosphatase inhibitors (100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM pepstatin A and 100 μM sodium pervanadate). Cells were solubilized for 30 minutes with rocking at 4^oC followed by centrifugation at 21,000xg. Supernatants were incubated for a total of 3-4 hours at 4°C with AP-4213 (8 μl), anti-Flag (10 μl) or AP-1/2 (2 μl) antibodies and 20 μl of a 50% slurry mixture of protein G agarose beads. Beads were then washed 3 times with TGH and denatured in

Laemmli buffer (2X) (250 mM Tris HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.01% Bromophenol Blue (w/v), 5% β-mercaptoethanol (v/v)). Proteins were separated on a 8% SDS-polyacrylamide gel and protein detection was assessed using AP-4213, β-adaptin, phosphor-ERK1/2 and ERK1/2 antibodies using conventional Western blot and chemiluminescence techniques according to the manufacturer's instructions (Perkin Elmer).

Immunofluorescence

HEK293 cells, seeded in 6 well plates ($0.15x10^6$ cells/well) with cover slips were transfected with Flag-β2-adaptin-YFP, and either HA-AT1R or EGFR or HA-AT1R and β2-adaptin shRNA or a scrambled shRNA. Cells were treated with Ang II (1 μM) and fixed with 4% (v/v) paraformaldehyde. Cells were washed 3 times with PBS containing 2% BSA and 0.05% triton (permeabilization buffer), followed by incubation for 45 minutes at room temperature with antibody AP-4213 (1:1000) in the permeabilization buffer. Cells were washed 3 times with permeabilization buffer, and then incubated with anti-rabbit Alexa fluor 568 (1:500) for 45 minutes at room temperature. This was followed by 3 washes with permeabilization buffer, and 3 washes with PBS alone. Cells were mounted onto slides using Geltol mounting medium (Thermo). Images were collected on a Zeiss LSM-510 Meta laser scanning microscope with a 40X oil immersion lens in a multitrack mode using a dual excitation (543 nm for Alexa fluor 568 and 514 nm for YFP or 488 nm for GFP) and emission (LP 560 nm for Alexa fluor 568 and BP 530-600 nm for YFP or BP 505-530 for GFP) filter sets.

Data Analysis

Intensity of the signals from Western blots was determined by densitometric analysis using MetaMorph Software (Universal Imaging Corporation), and is presented as the mean ± S.E.M. of at least three independent experiments.

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Figures and legends

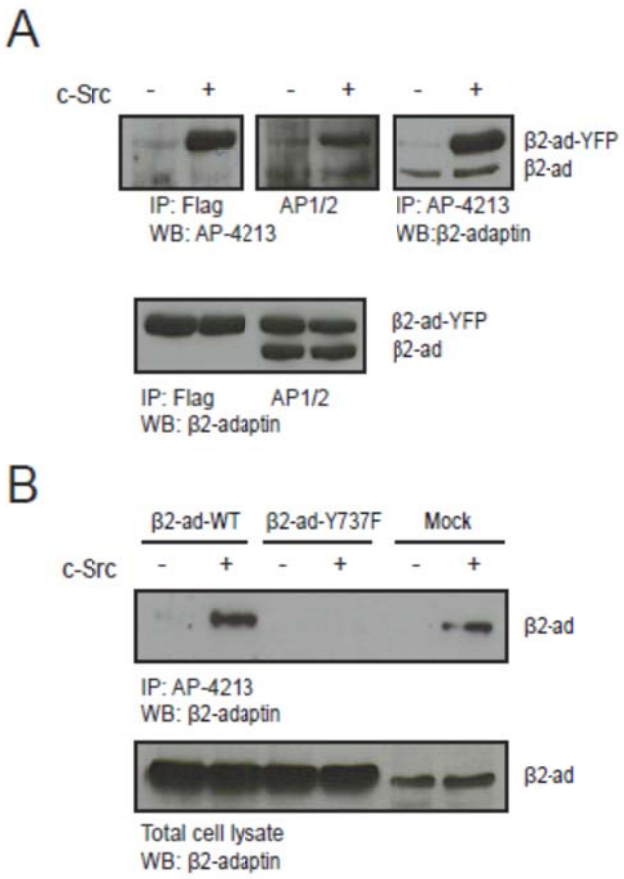


FIG 1. C-Src phosphorylates β2-adaptin on tyrosine 737 in cells

A, HEK293 cells were transiently transfected with Flag-β2-adaptin-YFP and either pcDNA3.1 (-) or Src kinase (+). Cell lysates were pooled, split into three fractions and immunoprecipitated with either anti-Flag, anti-AP1/2 or AP-4213 antibodies. The immunoprecipitates were analyzed by Western blot using an antibody directed against phosphotyrosine 737 in β2-adaptin (AP-4213) and an anti-β-adaptin antibody. Lower panel shows equal amount of β2-adaptin in the IP. B, HEK293 cells were transiently transfected with Flag-β2-adaptin, Flag-β2-adaptin-Y737F or pcDNA3.1 (mock). Cell lysates were immunoprecipitated with AP-4213 and were analyzed by Western blot using anti-β-adaptin antibody. Lower panel shows total β2-adaptin in total cell lysates.

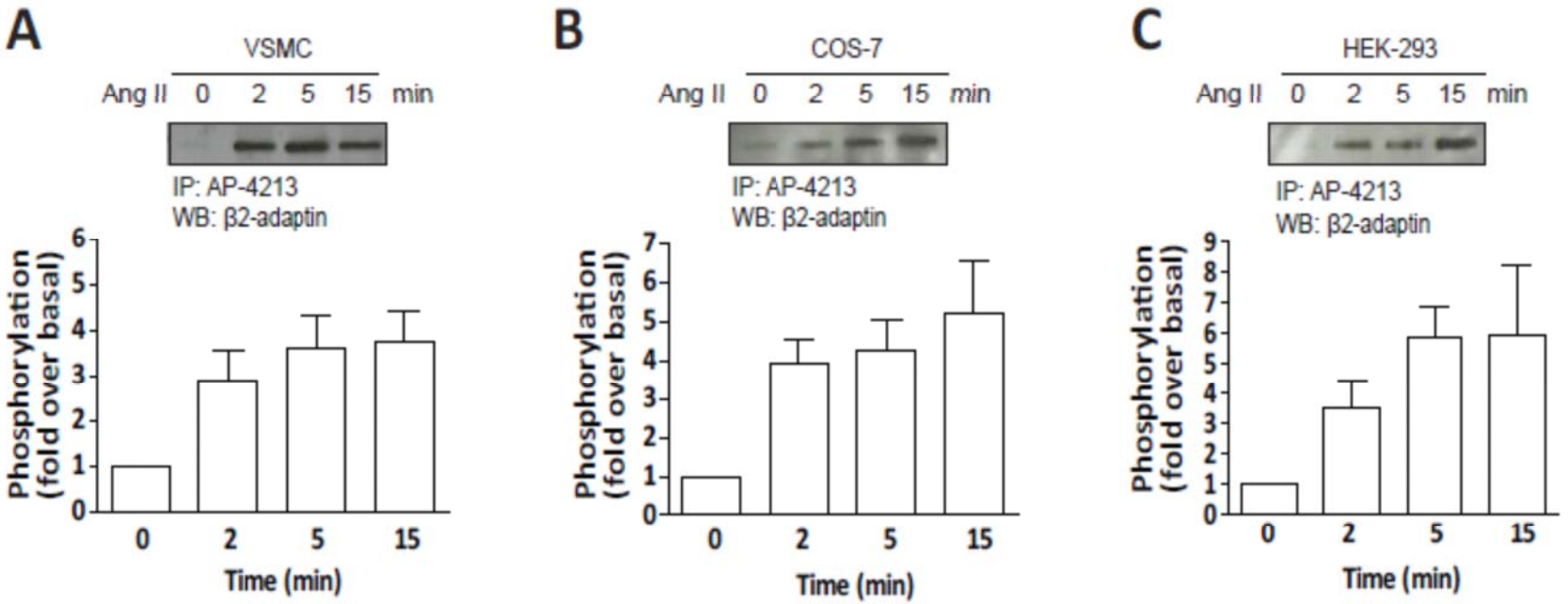


FIG 2. Ang II promotes the phosphorylation of tyrosine 737 of β2-adaptin in different cell types

A, VSMCs were serum-starved overnight and then stimulated with Ang II (1 µM) for the indicated time. Lysates were immunoprecipitated with AP-4213 antibody, and the immunoprecipitates were analyzed by Western blot using anti-β-adaptin antibody. B, COS-7 cells were transiently transfected with HA-AT1R, and then treated as described in A. C, HEK293 stably-expressing HA-AT1R were treated as described in A. Results are the mean ± S.E.M of at least 5 different experiments.

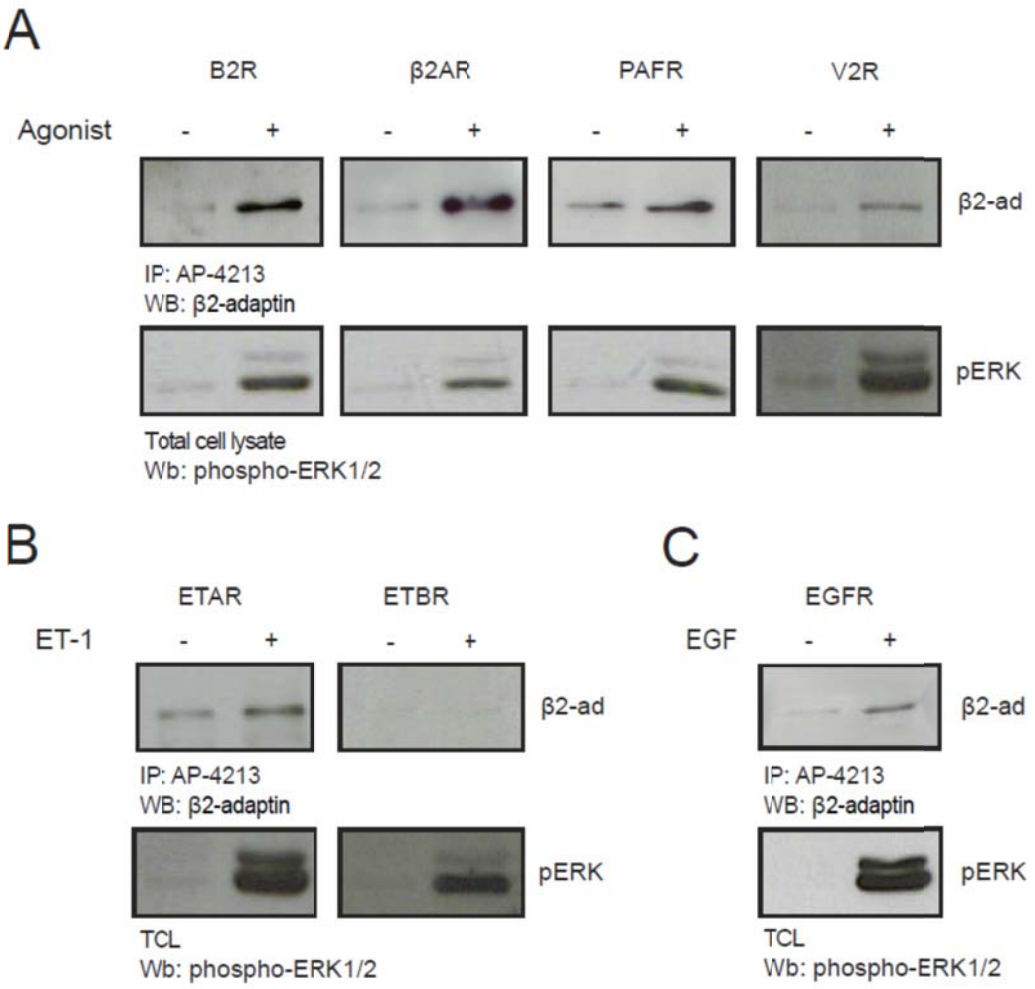
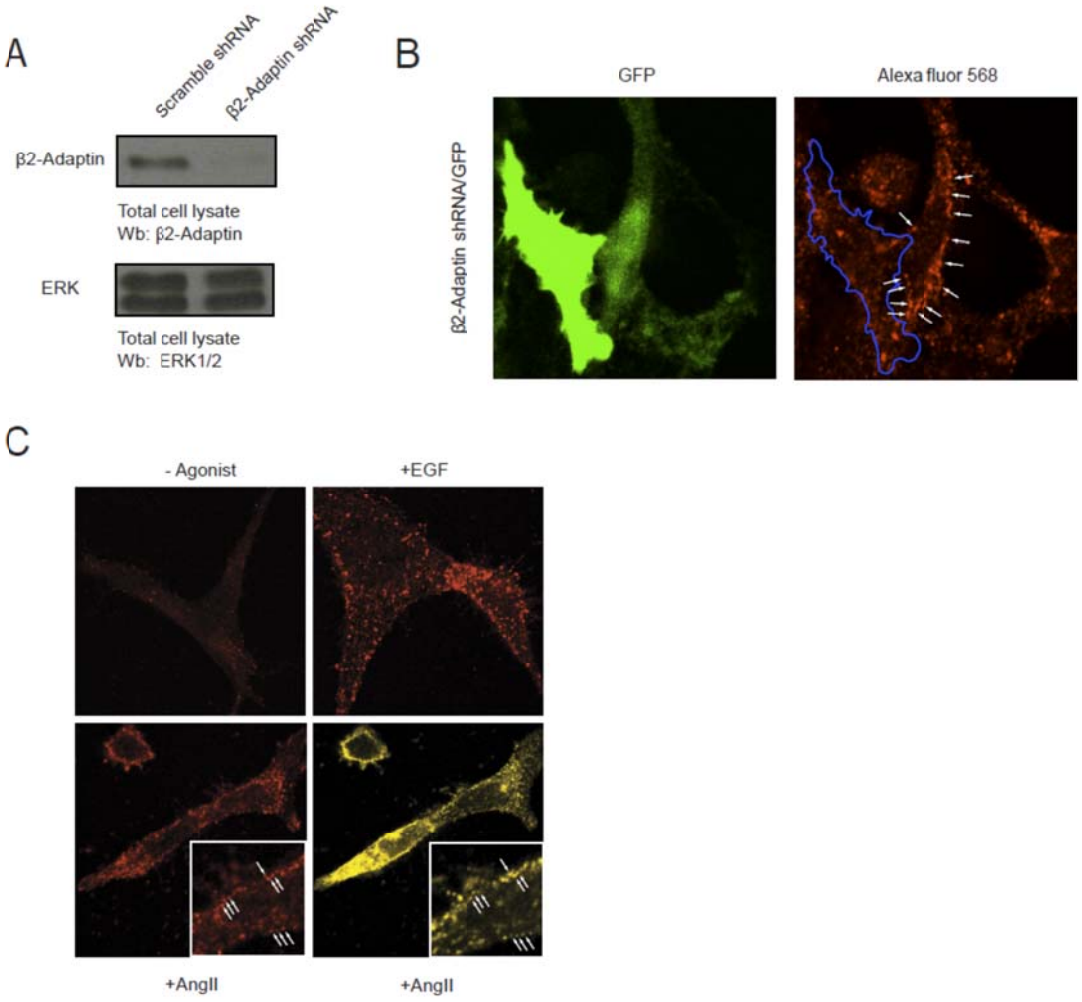

A
B2R
β2AR
PAFR
V2R
Agonist
- + - + - + - +
β2-ad
IP: AP-4213
WB: β2-adaptin
pERK
Total cell lysate
Wb: phospho-ERK1/2
B
ETAR
ETBR
ET-1
- + - +
β2-ad
IP: AP-4213
WB: β2-adaptin
pERK
TCL
Wb: phospho-ERK1/2
C
EGFR
EGF
- +
β2-ad
IP: AP-4213
WB: β2-adaptin
pERK
TCL
Wb: phospho-ERK1/2

FIG. 3. Other GPCRs and EGFR promote β2-adaptin tyrosine 737 phosphorylation

A, HEK293 cells were transiently transfected with the indicated receptor. Cells were serum-starved overnight and then treated with 1 μM of bradykinin, isoproterenol, carbamyl-PAF or arginine vasopressin, respectively, for 5 minutes (+) or were left untreated (-). Lysates were immunoprecipitated with AP-4213 antibody, and the immunoprecipitates were analyzed by Western blot using anti-β-adaptin and anti-phospho-ERK1/2 antibodies. Phospho-ERK1/2 was monitored to ensure that receptor activation had taken place. Blots are representative of at least 3 independent experiments. B, HEK293 cells were transiently transfected with either the endothelin type A (ETA) or type B (ETB) receptor. Cells were treated as in A, with endothelin (100 nM). Results are representative of at least 3 independent experiments. C, HEK293 cells were transfected with epidermal growth factor receptor (EGFR) and treated as in A with EGF (10 nM). Results are representative of 5 different experiments.


A
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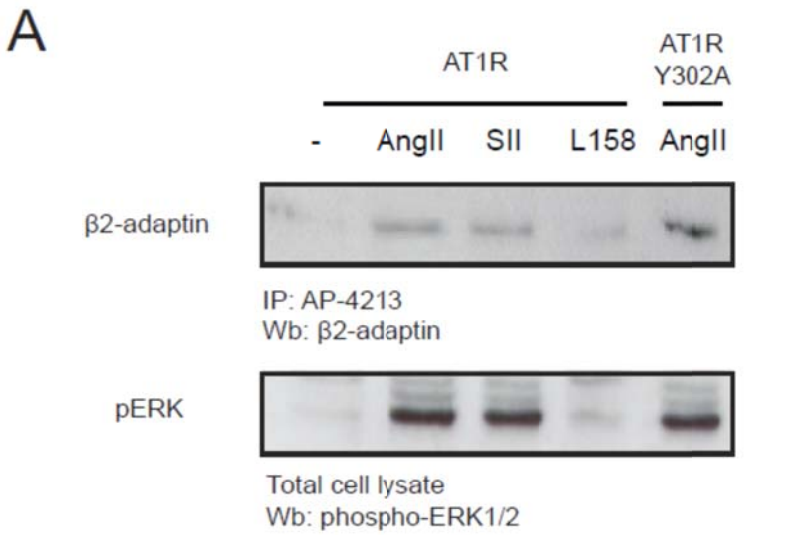
ERK
Total cell lysate
Wb: ERK1/2
B
GFP
Alexa fluor 568
β2-Adaptin shRNA/GFP
C
- Agonist
+EGF
+AngII
+AngII

FIG. 4. AT1R and EGFR promote the phosphorylation of β2-adaptin on Y737 in CCVs

A, HEK293 cells were transiently transfected with scrambled shRNA or shRNA against β2-adaptin. Cells were harvested 72 hours post transfection and lysates were analyzed by Western blot with a anti-β-adaptin antibody and an ERK1/2 antibody to ensure equal levels of protein loading. The blot is representative of 3 different experiments. B, HEK293 cells transiently transfected with HA-AT1R and β2-adaptin shRNA, which co-expresses GFP, were stimulated for 2 minutes with Ang II (1 μM) and were treated as indicated in the immunofluorescence protocol in Materials and Methods. High levels of GFP are correlated with high knockdown of β2-adaptin. The outline in blue in the right panel represents the plasma membrane of the cell with putative knockdown of β2-adaptin, whereas arrows indicate plasma membrane staining of AP-4213 in the cell with minimal β2-adaptin knockdown. Images are representative of 3 independent experiments C, HEK293 cells transiently transfected with EGFR or HA-AT1R and Flag-β2-adaptin-YFP were stimulated with EGF (10 nM) or Ang II (1 μM) for 2 minutes. Cells were treated as indicated in the immunofluorescence protocol in Materials and Methods. Arrows in insets indicate points of colocalization between β2-adaptin-YFP and AP-4213 staining. Images are representative of 3 independent experiments.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AT1R
AT1R Y302A
- AngII SII L158 AngII
β2-adaptin
IP: AP-4213
Wb: β2-adaptin
pERK
Total cell lysate
Wb: phospho-ERK1/2

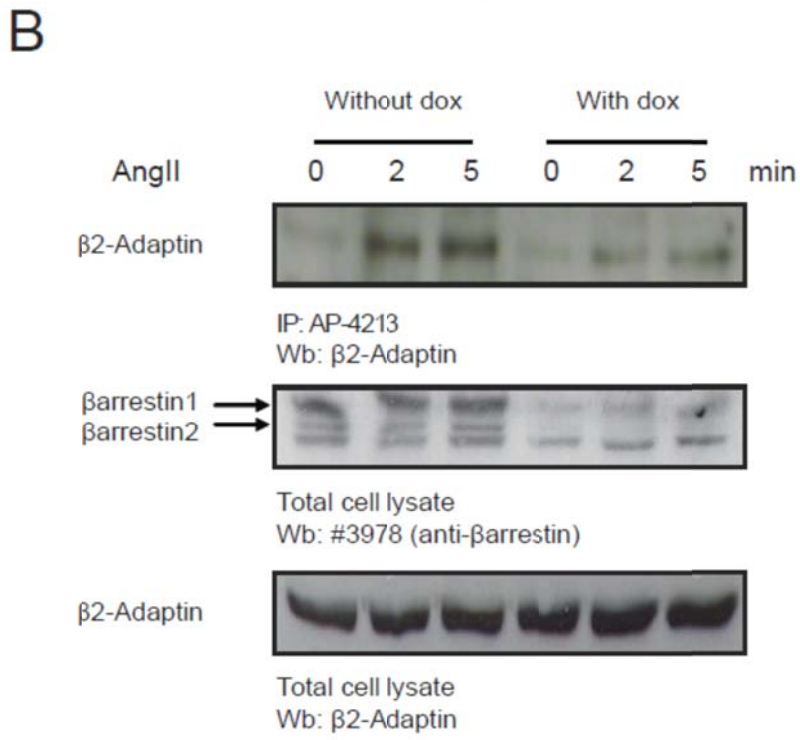
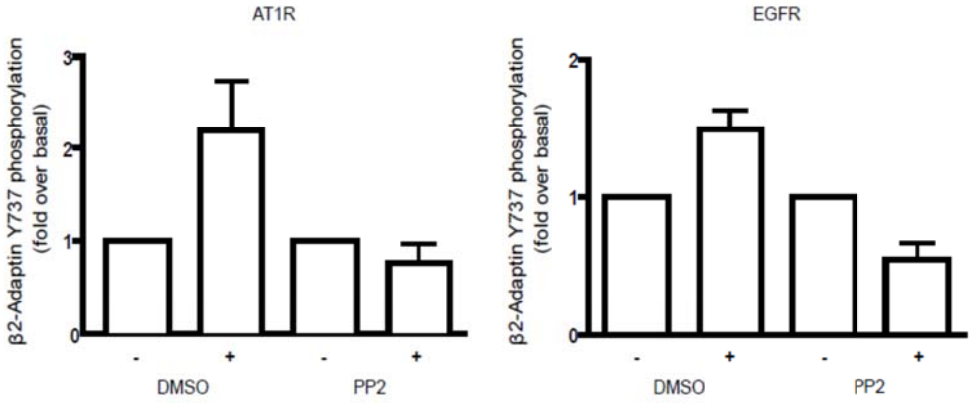

B
Without dox
With dox
AngII 0 2 5 0 2 5 min
β2-Adaptin
IP: AP-4213
Wb: β2-Adaptin
βarrestin1
βarrestin2
Total cell lysate
Wb: #3978 (anti-βarrestin)
β2-Adaptin
Total cell lysate
Wb: β2-Adaptin

FIG. 5. AT1R-mediated phosphorylation of AP-2 requires βarrestin

A, HEK293 cells were transiently transfected with HA-AT1R or HA-AT1R Y302A. The following day, cells were serum-starved overnight and then stimulated with either Ang II (1 μM), an angiotensin analog, $Sar^1Ile^4Ile^8$ (SII) (10 μM) or a non-peptidergic analog, L158,809 (10 μM) (L158). Lysates were immunoprecipitated with AP-4213 antibody, and the immunoprecipitates were analyzed by Western blot using anti-β-adaptin and phospho-ERK1/2 antibodies. Blots are representative of at least 3 independent experiments. B, HEK293 cells stably expressing inducible shRNA against both βarrestin1 and βarrestin2, were transiently transfected with AT1R. The following day, the media was replaced with full medium, with half the dishes receiving 1 μg/ml doxycycline (with dox), whereas the rest received only medium (without dox). Cells were induced for 72 hours post addition of doxycycline and were then stimulated with Ang II (1 μM). Lysates were treated as in A. Blots are representative of 4 independent experiments.

A


AT1R
EGFR
β2-Adaptin Y737 phosphorylation (fold over basal)
0
1
2
3
-
+
-
+
DMSO
PP2

B

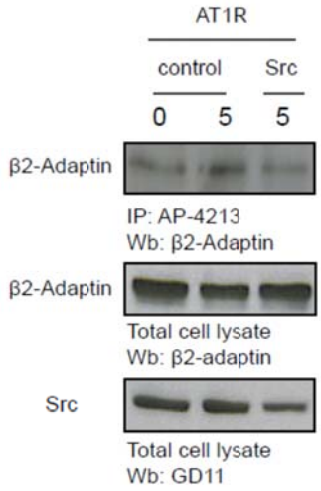

AT1R
control
Src
0
5
5
β2-Adaptin
IP: AP-4213
Wb: β2-Adaptin
β2-Adaptin
Total cell lysate
Wb: β2-adaptin
Src
Total cell lysate
Wb: GD11

FIG. 6. AT1R and EGFR-mediated AP-2 phosphorylation requires c-Src kinase activity

A, HEK293 cells were transiently transfected with HA-AT1R or EGFR. The following day, cells were serum-starved overnight. The next morning, cells were pretreated for 30 minutes with 5 μM PP2 or an equal volume of DMSO. Cells were then stimulated with Ang II (1 μM) for 5 minutes. Lysates were immunoprecipitated with AP-4213 antibody, and the immunoprecipitates were analyzed by Western blot using anti-β-adaptin antibody. Data are presented as the mean ± S.E.M. of three independent experiments. They represent the percent of β2-adaptin phosphorylation relative to its corresponding untreated point. B, HEK293 cells were transiently transfected with HA-AT1R and either non-silencing siRNA (control) or siRNA against c-Src (Src). 48 hours post transfection, cells were serum-starved overnight and the following morning stimulated with Ang II (1 μM) for 5 minutes. Lysates were immunoprecipitated with AP-4213 antibody, and the immunoprecipitates were analyzed by Western blot using anti-β-adaptin antibody. Lysates were blotted for anti-Src (GD11) and anti-β-adaptin antibodies.

Connecting text

The following section (**Chapter 3**) entitled, "A functional role for Y737 of β_2-adaptin in the signalling and endocytosis of the AT1R revealed through a single chain antibody" attempts to further characterize and establish a functional role for the phosphorylation of $\beta_2$adaptin in the AP-2 complex. In **Chapter 2**, we demonstrated that Y737 phosphorylation of $\beta_2$adaptin requires both βarrestin and Src, and that multiple stimuli can induce the event, however, we establish no functional role for the process. Attempts to determine a role were limited by the tools at our disposal, and early results found no significant impact on receptor internalization. However, as outlined in the following chapter, the development of a transfectable antibody targeted against pY737-$\beta_2$adaptin (intrabody) allowed for our better understanding of the importance of the site. As such, the main aims of our study were as follows:

1) Characterize the monoclonal antibodies generated to select candidates for single chain antibody synthesis,

2) Produce and determine the functionality of the single chain antibodies

3) Establish a role for $\beta_2$adaptin Y737 phosphorylation in signalling and endocytosis by using the transfectable single chain antibodies (scFv antibodies) as a means to disrupt the process

Chapter 3

A functional role for Y737 of β_2-adaptin in the signalling and endocytosis of the AT1R revealed through an intrabody

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Abstract

The regulation of clathrin-mediated endocytosis is complex and multi-faceted. Our previous work demonstrated that following activation of G protein-coupled receptors (GPCRs) like the angiotensin type 1 receptor (AT1R) or the receptor tyrosine kinase, epidermal growth factor receptor (EGFR), the β-subunit of the clathrin adaptor protein 2 (AP-2) complex ($\beta_2$adaptin) became phosphorylated on a specific tyrosine residue that was involved in the stability of the AP-2/βarrestin complex. However, we failed to establish a functional relevance to the phosphorylation event. Here we aimed to reveal a functional role of AP-2 phosphorylation in the context of receptor signalling or endocytosis, using a transfectable single chain antibody, which allowed for intracellular interference with the phosphorylated $\beta_2$adaptin. After verifying the functionality of the antibody against the site, we establish that binding of the antibody to tyrosine 737 phosphorylated-$\beta_2$adaptin blocks AT1R-mediated endocytosis. Furthermore, expression of the antibody halved receptor-mediated extracellular signal-regulated kinase (ERK) activation. Both of these results are supported by overexpression of a non-phosphorylatable $\beta_2$adaptin mutant. Our findings establish a novel mechanism to inhibit receptor endocytosis that allows for the targeted inhibition of agonist-activated receptors. Our results imply that tyrosine phosphorylation of Y737 on β2-adaptin is a common regulatory mechanism employed by different receptors undergoing clathrin-dependent endocytosis, and suggest a wider function for this event than originally anticipated.

Introduction

G protein-coupled receptors (GPCRs), integral membrane proteins containing seven transmembrane spanning regions, are responsible for controlling an array of biological processes such as phototransduction, olfaction, vascular tone, cardiac output and pain. The responsiveness of GPCRs to extracellular stimuli is multifactorial but relies largely on receptor expression and sensitivity. These aspects are modulated by receptor desensitization, internalization or endocytosis and resensitization, which involve the removal of refractory receptors from the plasma membrane, where they can be refreshed within the cell for reinsertion [1, 2]. Alternatively, receptor endocytosis can lead to continued intracellular signalling or the target degradation of the receptor to the lysosome. The most common route of GPCR internalization utilizes the clathrin pathway, where the central adaptors are βarrestin and the heterotetrameric clathrin adaptor protein 2 (AP-2) complex. Beta-arrestin targets the desensitized receptors to clathrin-coated pits (CCPs) through its interaction with the two major coat constituents; clathrin and AP-2, specifically the β-subunit, referred hereafter as $\beta_2$adaptin [3-6].

Accumulating evidence suggests that the regulation of clathrin-dependent internalization of GPCRs requires the recruitment of kinases and the phosphorylation of proteins within the clathrin coat [7-14]. Multiple studies have demonstrated that phosphorylation of dynamin by c-Src can regulate the internalization of the β_2-adrenergic or the epidermal growth factor (EGF) receptor [12, 14]. The heterotetrameric AP-2 complex represents a major target for phosphorylation with EGFR-dependent tyrosine phosphorylation on Y6 of β2-adaptin [13] and AAK-mediated threonine phosphorylation on T156 of μ_2-adaptin [11]. Our previous work also demonstrated βarrestin-dependent, Src-mediated tyrosine phosphorylation on Y737 of β2-adaptin that destabilized the βarrestin/AP-2 complex [9, 10]. Although we have identified Y737 as a regulatory

site, we have not established a functional role in terms of signalling or endocytosis following GPCR activation.

Single chain antibodies (scFv) are generated from the variable regions of the heavy and light chain immunoglobulins connected by a short 10-25 amino acid linker comprised of glycine, serine and threonine for flexibility and solubility [15]. The removal of the constant region does decrease the affinity for the antigen but specificity remains. A considerable benefit of single chain antibodies involves the ability to generate them directly from subcloned heavy and lights chains derived from a hybridoma [16]. Conventionally, scFvs are produced in bacteria, compared to monoclonal antibodies (mAbs) that are synthesized in mammalian cultures, however, as we have the antibody coding sequence, this allows for the generation of DNA constructs that can be transfected into cells, for scFv intracellular expression. This overcomes a major hurdle encountered with mAbs that target intracellular epitopes, as it is a difficult and often disruptive process to the cell to promote their entry. Multiple studies have demonstrated the benefits of intracellular scFvs, particularly in cancer. One study demonstrated that transfection of an antibody directed against a protein in cervical cancer, downregulated its expression levels and led to the inhibition of cell growth [17]. At a more mechanistic level, a scFv directed against ErbB2, a receptor whose signalling roles were unclear at the time, was used to prevent cell surface expression of the receptor through intracellular retention [18]. As such, while these antibodies may play a significant role therapeutically, they may also serve as effective tools to dissect signalling pathways and protein interactors.

To uncover the functional role of $\beta_2$adaptin Y737 phosphorylation in terms of receptor signalling and endocytosis, we developed a scFv against either phosphorylated or non-phosphorylated $\beta_2$adaptin. Here we reveal that expression of the anti-phospho-scFv halves receptor-mediated extracellular-signal regulated kinase (ERK) activation, while blocking receptor internalization

highlighting the critical role of $\beta_2$adaptin phosphorylation in proper receptor function.

Results

Based on our previous study where we generated a polyclonal antibody directed against phosphorylated tyrosine 737 in $\beta_2$adaptin of AP-2 (**Chapter 2**), we generated ten monoclonal antibodies directed against the same peptide fragment, GHIpYMEMN. Our goal was to eventually develop some of these clones into transfectable single chain antibody fragments. Based on the specificity data provided through ELISA analysis, eight of the ten clones generated specifically recognized the phosphorylated form of the peptide compared to the non-phosphorylated form (Table 1). The ELISA results suggested the most potent clone was 3D5E7 and at random we selected two other phosphorylation specific clones, 4B7A8 and 1C2B10, and one total $\beta_2$adaptin clone, 1E1F9, for screening by both immunoprecipitation and western blot. Unfortunately, clone 3D5E7 was ineffective at recognizing phosphorylated $\beta_2$adaptin compared to the non-phosphorylated form in both immunoprecipitation (figure 1A) and western blot (data now shown). However, clones 4B7A8 and 1C2B10 were much more promising with both functioning in immunoprecipitation and western blot (figure 1B and 1C). However, blotting with 1C2B10 often resulted in increased background signal, and it appeared to have some affinity for the non-phosphorylated form of $\beta_2$adaptin compared to 4B7A8. Immunoprecipitation with 1E1F9 gave similar results to commercially available antibodies against $\beta_2$adaptin recognizing both non-phosphorylated and phosphorylated forms equally (figure 1D).

Given the effectiveness of 4B7A8 and 1E1F9 in tests, these two clones were selected to have the coding sequence for the variable region of both the light and heavy chain isolated from the antibody generating hybridomas. The fragments were entered into sequentially into a vector; the heavy chain variable region followed by a 25 amino acid linker composed of glycine, serine and threonine and finally, the light chain variable region. Initial constructs featured a

C-terminal cyan fluorescence protein (CFP) tag to verify expression and to simplify isolation; however, future experiments revealed that this large moiety inhibited the interaction of the scFv with its target (data not shown). Instead we shuttled the construct into an N-terminal triple Myc vector, allowing for its detection and isolation with anti-myc antibody. In order to generate a reasonable control scFv for our studies, we reasoned that mixing the heavy chain of 1E1F9 and the light chain of 4B7A8 would generate a non-functional scFv that could not recognize $\beta_2$adaptin in either its phosphorylated or non-phosphorylated form, named scFv-FA. We proceeded to verify if the constructed we generated would be expressed. Lysates from mock transfected or appropriate 3xmyc-scFv-transfected cells were probed via western blot with anti-myc antibody revealing that all three construct did express a functional fusion protein. The AB7A8 construct migrates at ~44kDa, the 1E1F9 at ~40kDa and the chimera of the two migrates at ~42kDa (figure 2A).

We next assessed whether the expression of the scFvs in live cells allowed for their proper folding and function by assessing if they were capable of recognizing $\beta_2$adaptin, and if the 4B7A8 clone, maintained its specificity towards the phosphorylated form. Activation of the angiotensin type I receptor (AT1R), which we previously showed led to $\beta_2$adaptin Y737 phosphorylation, caused the interaction between scFv-4B7A8 and $\beta_2$adaptin to increase, whereas the interaction level was unaffected with scFv-1E1F9 (figure 2B).

We long suspected that interference with $\beta_2$adaptin in the heterotetrameric AP-2 complex, in particular the phosphorylated form, would disrupt the efficient internalization of the receptor. Our previous attempts had yielded no clear effect through knockdown studies, although this just removed the protein from the complex, perhaps allowing for adaptation by other processes. Given our ability to target complexed $\beta_2$adaptin when Y737 becomes phosphorylated, this allowed us to monitor the role for the event in terms of

receptor signalling and endocytosis in living cells. A radioactive ligand binding assay using ^{125}I-angiotensin II (AngII) on stably expressing HA-AT1R 293 cells transfected with mock vector, scFv-4B7A8, scFv-1E1F9, scFv-FA or dynamin-K44A (positive control for block) was performed. In cells transfected with either mock vector or scFv-FA, we saw an efficient, time-dependent internalization of the AT1R with the plateau for maximal internalization around 15 minutes of receptor stimulation (figure 3A), whereas there was a dramatically delayed endocytosis seen with dynamin-K44A expressing cells. However, in cells transfected with scFv-4B7A8, we saw a complete block of AT1R-mediated internalization, which is sustained, whereas the block mediated by scFv-1E1F9 (the general $\beta_2$adaptin antibody), becomes relieved after 15 minutes of receptor stimulation. This internalization block by scFv-4B7A8 was mimicked partially by overexpression of $\beta_2$adaptin-Y737F, a non-phosphorylatable form of $\beta_2$adaptin, although the effects are significantly less pronounced compared to the antibody, likely due to competition by endogenous wild-type $\beta_2$adaptin (figure 3B). To further verify our binding data, we performed a similar experiment using confocal microscopy on cells expressing AT1R-YFP and βarrestin2-mRFP as well as the scFv of interest or dynamin-K44A. Here we saw almost identical results with receptor in mock or scFv-FA transfected cells undergoing rapid endocytosis following AngII treatment (figure 3C), where the internalization in scFv-1E1F9 was delayed and in scFv-4B7A8 and dynamin-K44A cells no endocytosis has occurred after 15 min. Cells were followed up to 45min and no endocytosis was seen (data not shown).

Given the antibody's ability to affect endocytosis, we wondered whether signalling downstream of the receptor would also be affected. AT1R stably expressing cells were transfected with mock vector or the scFvs, and phosphorylation of ERK1/2 following AngII treatment was monitored via western blot. We observed a consistent 50% reduction in the level of ERK1/2 activation in cells expressing scFv-4B7A8 compared to both mock and scFv-FA transfected

cells (figure 4A). Surprisingly, expression of scFv-1E1F9 had no significant effect on ERK1/2 phosphorylation levels despite its inhibition of endocytosis. In our previous paper concerning the polyclonal antibody targeting Y737 of $\beta_2$adaptin, we demonstrated that other receptors including the epidermal growth factor receptor (EGFR) could induce Y737 phosphorylation. Therefore, we wondered whether the ERK1/2 activated by EGFR would also be affected by the scFv-4B7A8. Stimulation of endogenous EGFR in HEK293 cells with EGF also had a 50% block of the ERK1/2 phosphorylation (figure 4B). To support the role of Y737 phosphorylation in AT1R-mediated signalling, we also performed the experiments in the AT1R-stable cell-line while over-expressing empty vector, wildtype $\beta_2$adaptin or $\beta_2$adaptin-Y737F. Cells treated with AngII or EGF had about a 40% decrease in their levels of ERK1/2 activation in $\beta_2$adaptin-Y737F expressing cells compared to those with empty vector or wildtype $\beta_2$adaptin (figure 4C).

Phosphorylated tyrosines are often known to be molecular scaffolds for other proteins through src homology 2 (SH2) binding or phosphotyrosine binding (PTB) domains. Tyrosine 737 in $\beta_2$adaptin is followed by a methionine, glutamic acid and another methionine residue, which belongs to the consensus motif for binding (YxxM) of the regulatory subunit of PI3Kβ, p85. Therefore, we rationalized that perhaps the importance of $\beta_2$adaptin Y737 phosphorylation was not primarily to dissociate the βarrestin/AP-2 complex as we have demonstrated but that the dissociation was the result of recruitment of a secondary protein. Indeed, this could explain why the $\beta_2$adaptin-Y737F/βarrestin interaction is more stable, as no protein is recruited. Furthermore, it is difficult structurally to explain why phosphorylation at Y737 would disrupt the $\beta_2$adaptin/βarrestin binding, as the site of interaction is far from the phosphorylation event. To determine if the SH2 domain of p85 bound to Y737, we employed a GST-pulldown strategy using purified SH2 domain constructs and lysate containing non-phosphorylated or phosphorylated $\beta_2$adaptin. Five SH2 domain containing proteins were tested focusing on proteins shown to be involved in ERK1/2

signalling. Surprisingly, the SH2 domain with the highest relative avidity of those tested belonged to N-terminal SH2 of p120Ras-GAP, a key negative regulator of Ras-Raf-MEK-ERK activation (figure 5). We did find that both Shc and p85 also interacted to a lesser extent but both the C-terminal SH2 of p120Ras-GAP and the SH2 of Src did not interact, demonstrating stringency in our assay. This points to $\beta_2$adaptin being a molecular signalling scaffold as well as an endocytic adaptor, similar to what was discovered for βarrestin.

Discussion

Our results presented here reveal the importance of post-translational modification of the heterotetrameric AP-2 complex, specifically the β-subunit, in the signalling and endocytosis of GPCRs. We demonstrate that preventing $\beta_2$adaptin's tyrosine phosphorylation or masking the phosphorylation event with an intracellular antibody results in a dramatic block of receptor endocytosis and a halving of receptor-mediated ERK1/2 activation that was also recapitulated with a non-GPCR, the receptor tyrosine kinase, EGFR. We further demonstrate that Y737 on $\beta_2$adaptin is actually important for the recruitment of SH2 domain-containing proteins, including p120Ras-GAP, Shc and the regulatory subunit of PI3K, p85. Our findings establish a novel role for the endocytic adaptor, AP-2, one that includes serving as a molecular scaffold for the recruitment of proteins not only involved in endocytosis but also involved in the signalling output downstream of the receptor.

The complex process of clathrin-dependent internalization utilizes multiple adaptors proteins, some of which also play functional roles in receptor signalling. Previous work in our lab established that following activation of the AT1R, the receptor recruited βarrestin that led to the scaffolding of c-Src by βarrestin [9, 19]. AT1R/βarrestin complexes were then targeted to clathrin-coated pits through βarrestin's interaction with AP-2. Once in complex, Src phosphorylated AP-2 on its β-subunit specifically on tyrosine 737 [10], which was critical for the efficient dissociation of the AP-2/βarrestin complex. Despite the relevance of phosphorylation in the stability of the complex, no clear functional output was attributed to the event. Here using an intracellular single chain antibody directed against pY737 $\beta_2$adaptin, we revealed the importance of the site in both the endocytosis and downstream signalling of the AT1R, as both processes were significant disrupted by the antibody. Furthermore, our confocal results revealed that while the receptors still traffic to clathrin-coated pits, they

are retained at this stage, likely due to improper dissociation of the AT1R/βarrestin/AP-2 complex as a result of the antibody.

In our previous study, we demonstrated using a polyclonal antibody directed against phosphorylated Y737 in $\beta_2$adaptin that Y737 phosphorylation was a common occurrence among numerous GPCRs undergoing clathrin-mediated endocytosis but also that a non-GPCR like EGFR induced the phosphorylation as well. Conceptually, this pointed to a more significant role for the phosphorylation event than solely dissociating the AP-2/βarrestin complex but with the tools available during our first study, we were unable to concretely establish a function. With the production of the single chain antibodies we established that this tyrosine residue when phosphorylated appears to act as a docking area for the SH2 domains of proteins involved in critical signalling pathways downstream of GPCRs. Indeed we show that using the scFv to latch onto the phosphorylation site or a $\beta_2$adaptin-mutant that is phosphorylation deficient, this leads to a 50% reduction in the agonist-mediated ERK1/2 activation downstream of both the AT1R and EGFR. Furthermore, using the SH2-domains from multiple signalling proteins, we reveal that $\beta_2$adaptin is capable of a strong interaction with the N-terminal SH2 domain of p120Ras-GAP and slightly weaker interactions with the Shc and p85β SH2.

The interaction of p120Ras-GAP is surprising given our results, as it is a negative regulator of ERK1/2 signalling [20]. Presumably, blocking its interaction with $\beta_2$adaptin should increase receptor-mediated signalling. Despite this, p120Ras-GAP may play a role here in the endocytic regulation of the receptor, as it has been shown to be in complex with EGFR during its endocytosis and at the endosomal level [21]. Furthermore, p120Ras-GAP has been shown to be critical for proper recycling of integrin receptors [22] and has been shown to be a potential GAP for Rab5, which is found on clathrin-coated vesicles and early endosomes [23]. While recruitment of p120Ras-GAP to the plasma membrane

following receptor tyrosine kinase activation is known, the protein responsible for the recruitment is not; therefore it is plausible AP-2, which is also found in these internalization complexes, could be this protein. Potentially, the absence of p120Ras-GAP in the complex could prevent proper cycling of the Rab5, leading to an endocytic block.

The potential for Shc and p85β to be involved in the regulation of ERK signalling through Y737 of $\beta_2$adaptin is more likely due to their roles as signal potentiators. The SH2 domain in Shc binds to pY-E/I-x-I/L/M motifs [24], while p85β binds to pY-x-x-M motifs preferentially. Tyrosine 737 of $\beta_2$adaptin, perfectly mimics a p85β binding motif with pYMEM, however, Shc interacts to a similar degree. Both proteins are found upstream of ERK activation, with Shc using being a primary adaptor to a receptor tyrosine kinase, which leads to Grb2/SOS recruitment and subsequent Ras activation, leading to the Raf-MEK-ERK cascade [25, 26]. The involvement of p85β in ERK activation has been demonstrated using PI3K inhibitors like wortmannin, which block agonist-induced ERK phosphorylation [27]. Interestingly, studies have shown that Src-mediated phosphorylation is required for Shc recruitment and activity, whereas other Src mediated-phosphorylation events were required for p85β/p110-PI3K recruitment, similar to what we are proposing [28].

While we established previously that $\beta_2$adaptin Y737 phosphorylation was an important regulator of the AP-2/βarrestin complex stability, the reason for its involvement was unclear. Structurally, the interaction of $\beta_2$adaptin and βarrestin occurs at the top of the appendage domain of $\beta_2$adaptin [5]. Y737 in $\beta_2$adaptin is found near the end of the flexible hinge domain, on the exterior of the protein, making it unlikely to dramatically affect protein folding due to steric or charge interactions. However, given its exposure to the cytosol, it does serve as a perfect location for a scaffolding site. Indeed, it is plausible that the dissociation of $\beta_2$adaptin from βarrestin occurs when a large protein is recruited

on this phosphorylated tyrosine, leading to steric interference with the bound βarrestin. Functionally, this is the most logical explanation to explain why substitution of the tyrosine for a phenylalanine delays AP-2/βarrestin separation.

While this single chain antibody is an excellent tool for studying the functional roles of $\beta_2$adaptin tyrosine phosphorylation on receptor-mediated signalling and endocytosis, it also may be useful therapeutically. Single chain antibodies have been shown to be beneficial in multiple studies at targeting specific proteins involved in cancer often inhibiting cancer progression. One scFv directed against the intracellular domain of ErbB2 resulted in the downregulation of the receptor on the cell surface, and beneficially induced the apoptosis of ErbB2-overexpressing ovarian cancer cells [29]. Another intrabody, which was directed against the folate receptor, led to the receptor downregulation and reversion of the ovarian cancer cell phenotype [30]. Our single chain antibody directed against pY737 $\beta_2$adaptin may also be of therapeutic interest in cancer, as two large scale phosphoproteomic surveys identified this site as being hyperphosphorylated in Src-transformed cell lines and EGFR-dependent tumors [31, 32]. Amazingly, this was the only tyrosine among the over 30 found in $\beta_2$adaptin to be hyperphosphorylated, suggesting it may have a functional significance in cancer cell progression, perhaps due to aberrant regulation of both signalling and endocytosis of membrane bound receptors. Future work with scFv-4B7A8 will examine the effects of intrabody expression on the phenotype of different cancer cell populations.

Materials and methods

Materials

Angiotensin II, trichloroacetic acid (TCA) and poly-l-lysine are from Sigma Chemical Co. Sar_1,Ile_8-AngII (SI) is from MP Biomedicals. Epidermal growth factor (EGF) is from Fitzgerald Industries International (Acton, MA). Antibodies against pERK1/2 and total ERK1/2 were from Cell Signalling Technology. Anti-βadaptin antibody was from BD Biosciences. Anti-AP2, anti-flag, anti-myc, anti-mouse and anti-rabbit HRP-conjugated IgG were from Sigma. Western Lightning® Plus–ECL and ^{125}-Iodine were obtained from Perkin-Elmer. Pre-coated iodination tubes (Thermo Scientific) and acetonitrile were purchased for Fisher Scientific. Sep-Pak® Plus C18 cartridges are from Waters. Glutathione sepharose 4B beads are from GE Amersham. Protein G agarose is from Roche. Monoclonal antibodies were synthesized by Genscript at our request. They also isolated the cDNA sequences of the heavy and light variable regions.

Constructs

AT1R-YFP, βarrestin2-mRFP, Src-250-536, Flag-β2-adaptin, siRNA insensitive Flag-β2-adaptin-YFP and Flag-β2-adaptin-Y737F-YFP mutants were described elsewhere [10]. The 3xmyc vector used to clone the scFvs was a generous gift from Dr. Jean-Phillipe Gratton. The single chain antibody for 4A7B8 was inserted into a backbone vector containing to insertable regions with a short linker separating the two regions. The enzymes flacking each region were EcoRV/KpnI and NheI/XhoI. The heavy and light chains were inserted sequentially, using primers with appropriate restriction enzymes. A C-terminal RlucII was present originally, but it was swapped out for a sCFP-STII (streptavidin II). The PCR clonings were performed using either the Infusion system (Clonetech) or CloneEz (Genscript). The 1E1F9 and scFv-FA were generated by switching out the variable regions from the 4A7B8 construct. Finally, all scFvs were transferred into

the pCMV3T-2A (3xmyc) vector from Stratagene digested with EcoRV/XbaI, while the scFvs were digestedwith EcoRV/XhoI.

Cell culture and transfection

Human Embryonic Kidney (HEK293) cells stabling expressing the HA-AT1R were grown in MEM (Hyclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) and gentamicin (100 µg/ml, Gibco). Cells seeded in a 100-mm dish, at a density of $1.2x10^6$ cells per dish, were transfected using a calcium phosphate co-precipitation method as previously described [19]. All experiments were performed 48 hours post-transfection.

GST purification protocol

BL21 bacteria expressing the various SH2 domains were grown overnight in a small volume (~5ml) of LB @ 37°C. The next morning cells are diluted to an OD_{600} of 0.1 and allowed to grow until the OD reaches ~0.6. Cells were then induced with 1mM IPTG for 3 hours @ 30°C. Bacteria were then pelleted and frozen. GST proteins were then isolated by resuspending cells in cold PBS (pH7.4) containing 2mM EDTA, 4mM PMSF, 1% triton X-100 (v/v) and 1mg/ml lysozyme. Cells were sonicated on ice followed by 3 freeze-thaw cycles with liquid nitrogen. 75µl of glutathione-sepharose 4B beads were added to each lysates, rocked for 2 h at 4°C, and then washed 5 times with PBS. GST fusion proteins were analyzed by SDS-PAGE and protein concentration was determined using a DC protein assay kit (Bio-Rad).

Immunoprecipitation and western blot experiments

HEK293 cells were serum-starved overnight, and the next day incubated for 30 minutes at 37°C in their respective media, buffered with 20 mM HEPES pH 7.4. Cells were then treated with Angiotensin II (Ang II) (1 µM) for the indicated period of time. Stimulation was stopped on ice, washed with ice-cold PBS, and

cells were solubilized in TGH buffer (50 mM HEPES pH 7.4, 1% Triton X-100 (v/v), 10% glycerol (v/v), 50 mM NaCl, 5 mM EDTA) containing protease and phosphatase inhibitors (100 µM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM pepstatin A and 100 µM sodium pervanadate). Cells were solubilized for 30 minutes with rocking at 4^{o}C followed by centrifugation at 21,000xg. Supernatants were incubated for a total of 3-4 hours at 4°C with AP-4213 (8 µl), anti-Flag (5 µl), anti-myc (1µl) or AP-1/2 (1 µl) antibodies and 20 µl of a 50% slurry mixture of protein G agarose beads. For GST pulldown experiments, 2.5µg of GST fusion protein coupled to beads were used in place of antibodies. Beads were then washed 3 times with TGH and denatured in Laemmli buffer (2X) (250 mM Tris HCl pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.01% Bromophenol Blue (w/v), 5% β-mercaptoethanol (v/v)). Proteins were separated on a 8% SDS-polyacrylamide gel and protein detection was assessed using β-adaptin, anti-myc, 4B7A8, 1E1F9, 1C2B10, phospho-ERK1/2 and ERK1/2 antibodies using conventional Western blot and chemiluminescence techniques according to the manufacturer's instructions (Perkin Elmer).

Labeling of tracer and K_d determination

^{125}I- Angiotensin was generated as previously described [33].

Radioactive ligand binding internalization assay

HEK293 cells stably expressing the HA-AT1R were plated in 10cm dishes. 24 h later, cells were transfected with lipofectamine 2000 and the appropriate construct (mock or scFv). 24 h post-transfection, cells were resplit into 24-well plates coated with poly-L-lysine. The following morning, cells were serum-starved in MEM containing 20mM HEPES for at least 10 h prior to commencing the experiment. Cells were then treated with 100nM AngII for the different amounts of time at 37°C. Cells were then moved to room temperature, where they were washed four times for 5min with PBS. 400µl of ice-cold PBS was then

added to non-specific conditions, with 450µl being added to specific conditions and cells were put on ice. Non-specific wells had 50µl of PBS containing AngII (final concentration = 1µM). Every well received 50µl of ice-cold PBS containing 0.5µCi of ^{125}I-AngII (1µCi/µl) and were incubated overnight. The following morning, cells were washed 3 times with ice-cold PBS, and then resuspended in 500µl of 1M NaOH. The radioligand content was evaluated by γ-counting on a Perkin Elmer Wizard 1470 automatic gamma counter.

Live cell imaging

AT1R-YFP and βarrestin2-mRFP were transfected in HEK293 cells plated in 35mm dishes (Mattek), and 24 hours later, cells were serum-starved overnight. The following morning, fresh media containing 20mM HEPES was used and cells were further starved 30 min. Cells were then stimulated with 1µM AngII and monitored live for up to 45 min. Images were collected at 7 different fields during each dish with images usually being taken every 60s each field for the first 15 minutes and then at larger intervals after. A 63X oil immersion lens in multi-track mode using excitation at 514nm for YFP and 543nm for mRFP, with emission being collected using the BP530-600 (YFP) and LP560 (mRFP) filter sets.

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Figures and legends

Table 1. ELISA results using the 10 monoclonal antibodies generated by Genscript. Different dilution of antibody in columns showing relative affinity of antibody for the non-phosphopeptide or phosphopeptide

	Dilution	1:10	1:90	1:270	1:2,430	Titer
3D5E7	phospho-peptide	2.455	2.438	1.965	0.626	>1:2,430
	non-phospho-peptide	0.091	0.093	0.087	0.105	0
4B7A8	phospho-peptide	2.088	1.823	1.133	0.469	>1:2,430
	non-phospho-peptide	0.087	0.087	0.097	0.076	0
1C2B10	phospho-peptide	2.247	2.007	1.394	0.57	>1:2,430
	non-phospho-peptide	0.093	0.081	0.095	0.088	0
1E1F9	phospho-peptide	2.452	2.334	2.164	1.117	>1:2,430
	non-phospho-peptide	2.539	2.406	2.127	1.568	>1:2,430
1C2D9	phospho-peptide	2.351	1.939	1.223	0.455	>1:2,430
	non-phospho-peptide	0.102	0.109	0.095	0.107	0
1E1D7	phospho-peptide	2.295	2.27	2.239	1.057	>1:2,430
	non-phospho-peptide	2.319	2.272	2.245	1.258	>1:2,430
3A7C6	phospho-peptide	1.871	1.3	0.0492	0.242	>1:2,430
	non-phospho-peptide	0.106	0.091	0.086	0.1	0
3A7E5	phospho-peptide	2.047	2.072	0.866	0.2	>1:2,430
	non-phospho-peptide	0.091	0.086	0.11	0.124	0
3D5B9	phospho-peptide	2.364	2.103	1.611	0.49	>1:2,430
	non-phospho-peptide	0.18	0.076	0.074	0.079	>1:10
4B7A2	phospho-peptide	1.998	1.507	1.057	0.386	>1:2,430
	non-phospho-peptide	0.118	0.084	0.076	0.076	0

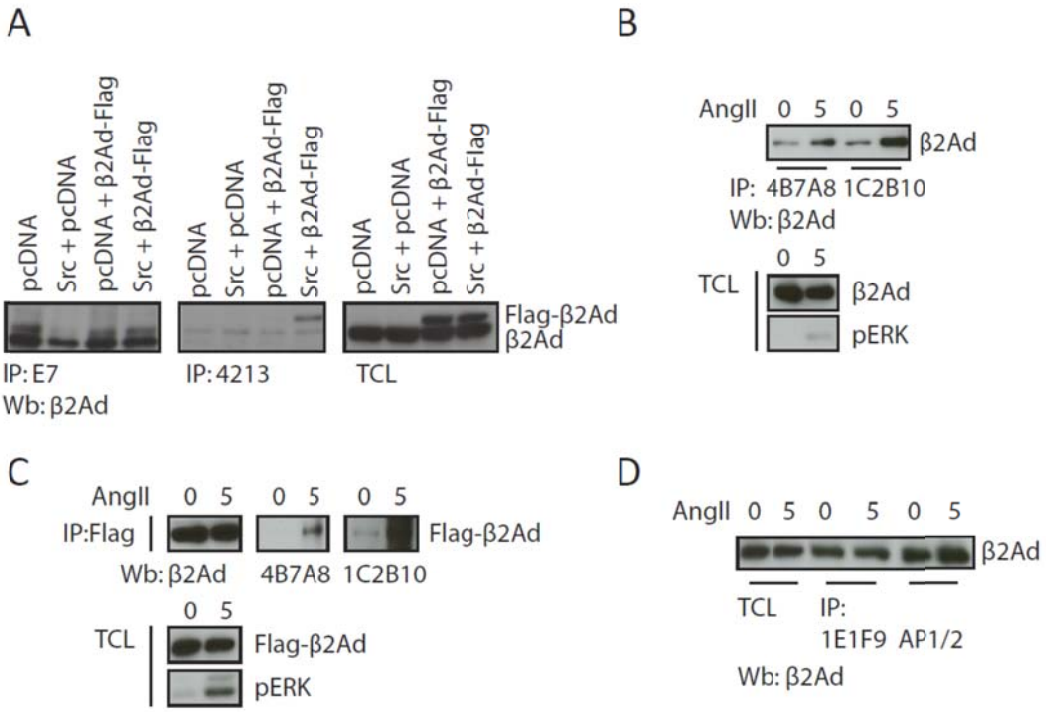

Figure 1. Characterization of 3D5E7, 4B7A8, 1C2B10 and 1E1F9 monoclonal antibodies.

A, HEK293 ells transfected with β2Ad-Flag or empty vector and Src or empty vector were immunoprecipitated with 3D5E7 or AP-4213. Immunoprecipitates were run on an SDS-PAGE gel and blotted for β2Adaptin. Blots are representative of 4 independent experiments. B, HA-AT1R stably expressing cells were stimulated for 5 minutes and then immunoprecipitate with 4B7A8 or 1C2B10. Immunoprecipitates were run on SDS-PAGE and blotted for β2Adaptin. Blots are representative of 5 independent experiments. C, Cells were treated as in B, except were also transfected with β2Adaptin-Flag and immunoprecipitated with the anti-Flag antibody. After SDS-PAGE, immunoblot was performed using anti-β2Adaptin, 4B7A8 and 1C2B10. Blots are representative of 3 independent experiments. D, Cells were treated as in B, except were immunoprecipitated with 1E1F9 or AP1/2 (commercially available anti-adaptin antibody). Immunoblot was performed using another β2Adaptin antibody. Blots are representative of 3 independent experiments.

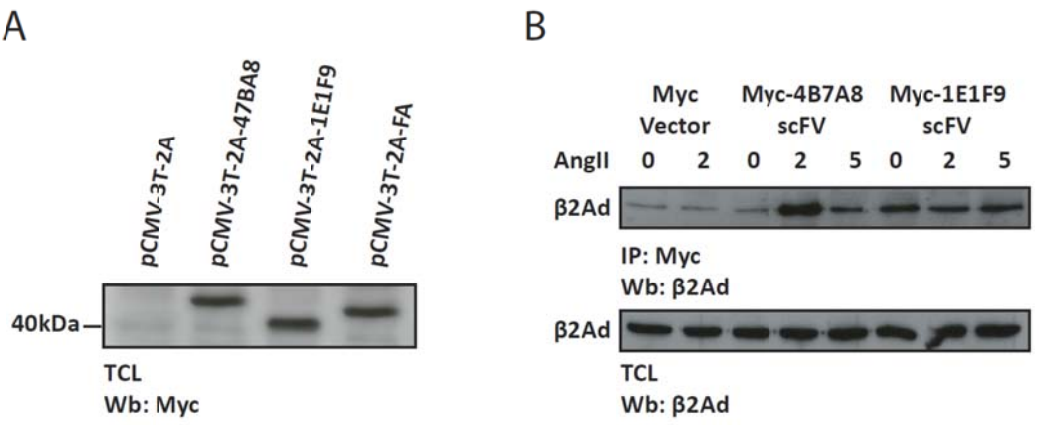

Figure 2. Expression and functionality of the single chain antibodies.

A, HA-AT1R stably expressing HEK293 cells were transfected with the indicated constructed. Lysates were prepared, run on SDS-PAGE and immunoblotted using the anti-Myc antibody. Blot is representative of 3 independent experiments. B. As in A, but cells were stimulated for the time indicated. Lysates were immunoprecipitate with anti-myc antibody to capture the scFv and whatever it was bound to it. Immunoprecipitates were run and immunoblotted for β2Adaptin. Blot is representative of 3 independent experiments.

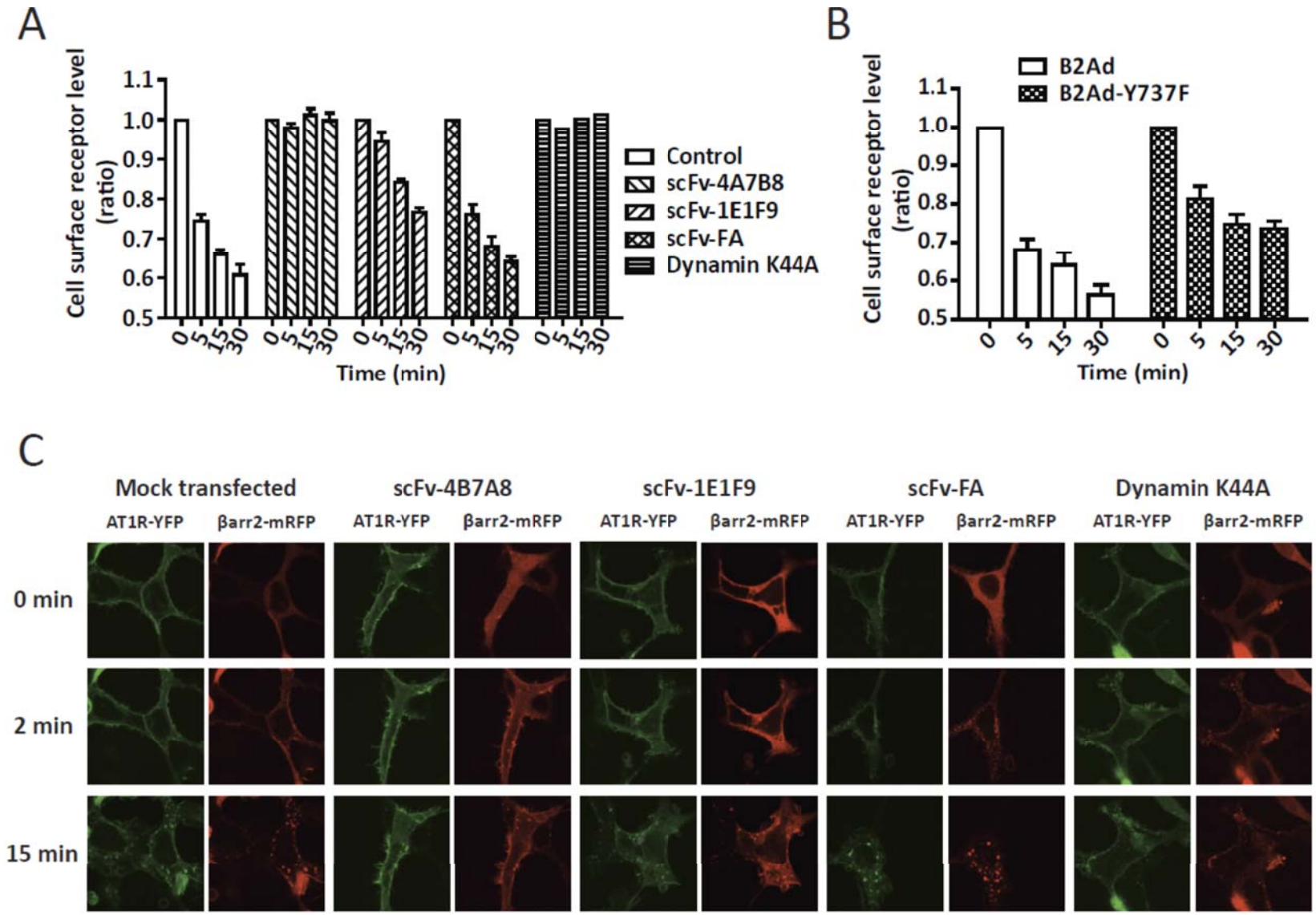
A
Cell surface receptor level (ratio)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0 5 15 30 0 5 15 30 0 5 15 30 0 5 15 30 0 5 15 30
Time (min)
Control
scFv-4A7B8
scFv-1E1F9
scFv-FA
Dynamin K44A
B
B2Ad
B2Ad-Y737F
Cell surface receptor level (ratio)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0 5 15 30 0 5 15 30
Time (min)
C
Mock transfected
scFv-4B7A8
scFv-1E1F9
scFv-FA
Dynamin K44A
AT1R-YFP
βarr2-mRFP
0 min
2 min
15 min

Figure 3. Endocytic block mediated by scFv-4B7A8 and the role of Y737 in internalization.

A, HEK293 cells stably expressing HA-AT1R were transfected with the construct indicated. 48h later, cells were treated with 100nM AngII for the indicated time. Cells were washed with PBS and incubated overnight with ^{125}I-AngII (1µCi/ml). The following morning cells were washed with ice-cold PBS to remove unbound and cells were harvested in 1M NaOH to count radioactivity bound. Data is presented as percent cell surface receptor remaining. Quantification is of 4 independent experiments performed in triplicate. B, As in A, except cells were transfected with β2Adaptin-wt or β2Adaptin-Y737F. Quantification is of 3 independent experiments performed in triplicate. C, HEK293 cells were transfected with AT1R-YFP and βarrestin2-mRFP. 30h later, cells were serum-starved overnight. The following morning, fresh serum-free media was used containing 20mM HEPES. 30min later, cells were stimulated with 1µM AngII for the indicated period of time, and were followed for up to 45min. AT1R-YFP is presented in pseudocolour green. Images are representative of 3 independent experiments performed in duplicate, with at least 3 cells were condition per plate.

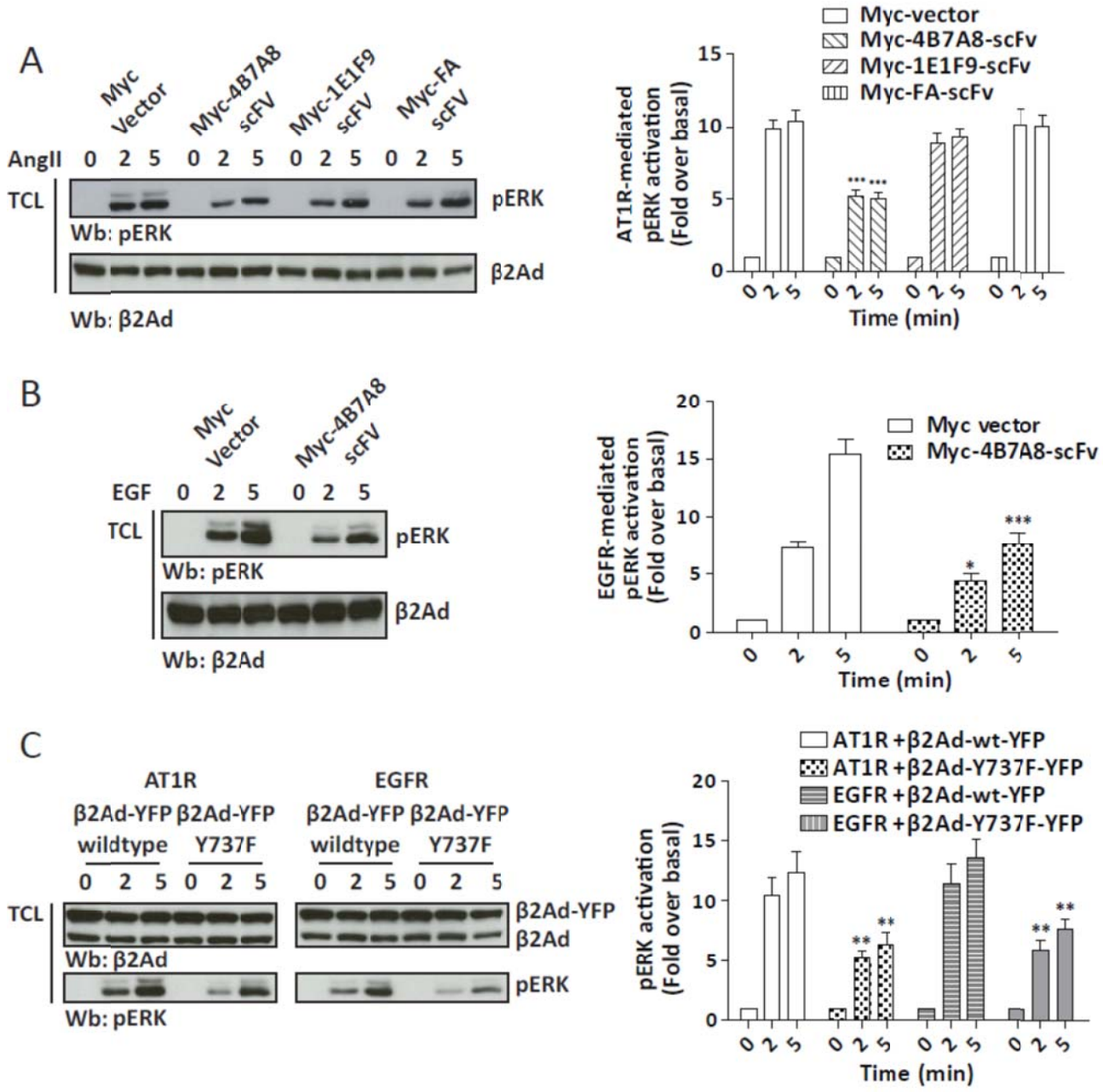
A
Myc Vector
Myc-4B7A8 scFV
Myc-1E1F9 scFV
Myc-FA scFV
AngII 0 2 5 0 2 5 0 2 5 0 2 5
TCL
pERK
Wb: pERK
β2Ad
Wb: β2Ad
Myc-vector
Myc-4B7A8-scFv
Myc-1E1F9-scFv
Myc-FA-scFv
AT1R-mediated pERK activation (Fold over basal)
Time (min)
B
Myc Vector
Myc-4B7A8 scFV
EGF 0 2 5 0 2 5
TCL
pERK
Wb: pERK
β2Ad
Wb: β2Ad
Myc vector
Myc-4B7A8-scFv
EGFR-mediated pERK activation (Fold over basal)
Time (min)
C
AT1R
EGFR
β2Ad-YFP β2Ad-YFP
wildtype Y737F
0 2 5 0 2 5 0 2 5 0 2 5
TCL
β2Ad-YFP
β2Ad
Wb: β2Ad
pERK
Wb: pERK
AT1R +β2Ad-wt-YFP
AT1R +β2Ad-Y737F-YFP
EGFR +β2Ad-wt-YFP
EGFR +β2Ad-Y737F-YFP
pERK activation (Fold over basal)
Time (min)

Figure 4. Role of Y737 phosphorylation in receptor mediated ERK1/2 activation.

A, HEK293 cells stably expressing HA-AT1R were transfected with the indicated construct. Cells were serum-starved overnight and the following morning media was replaced fresh. 30min later, cells were stimulated with 1µM AngII for the time indicated. Lysates were collected and run on SDS-PAGE and blotted for pERK and β2Adaptin. Quantification of 4 independent experiments (mean ± SEM) is shown. B, As in A, except cells were stimulated with 5ng/ml of EGF for the time indicated. Quantification of 3 independent experiments is shown. C, As in A, except cells were transfected with β2Adaptin-YFP wildtype or β2Adaptin-YFP-Y737F. Cells were treated with 1µM AngII or 5ng/ml EGF for the time indicated. Quantification is the result of 3 independent experiments. Statistical analysis was performed using two-way ANOVA followed by a post-hoc Bonferroni test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

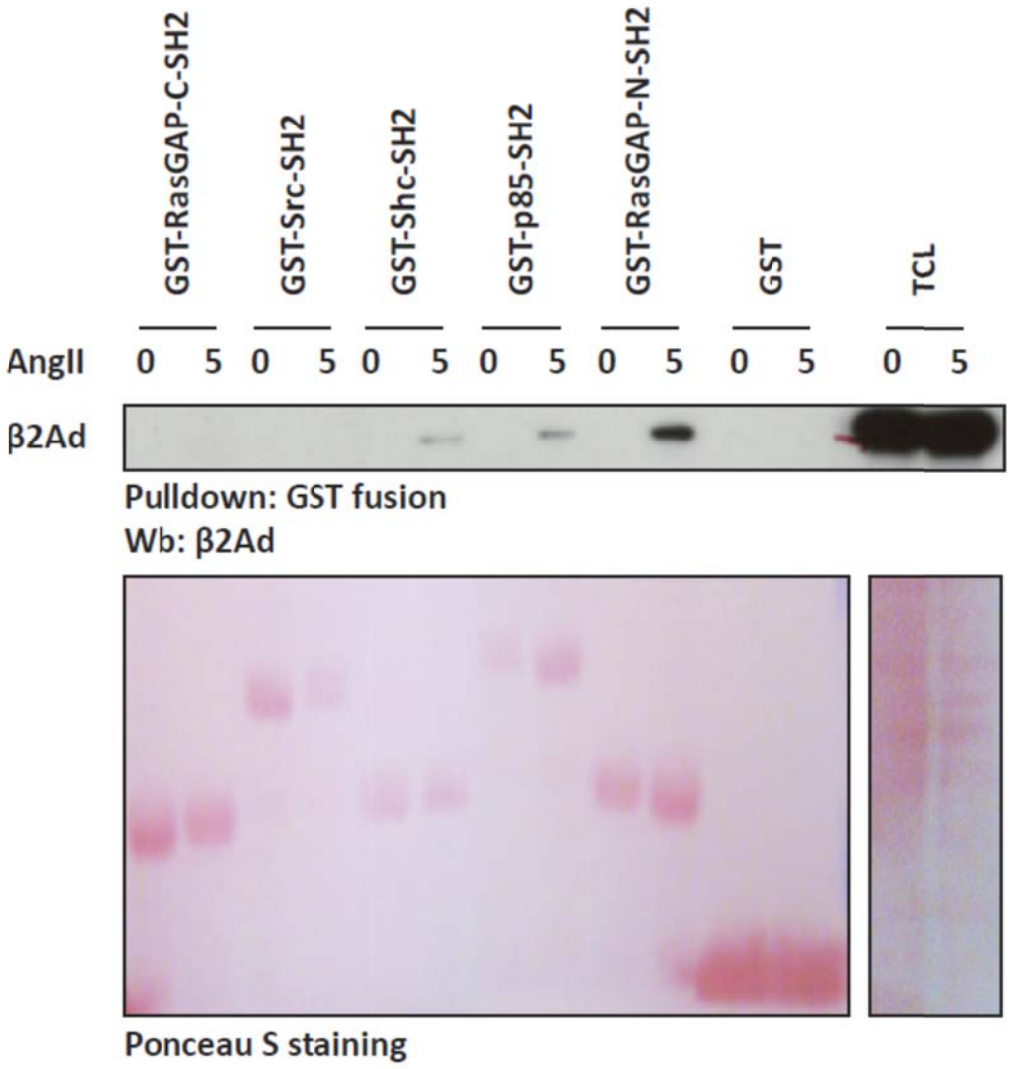

Figure 5. Interaction of phosphorylated $\beta_2$adaptin with SH2 domains of regulators of ERK1/2 signalling.

HEK293 cells stably expressing HA-AT1R cells were stimulated for the time indicated. Lysates were incubated with the indicated proteins' SH2 domain fused to GST. GST beads were isolated and run on SDS-PAGE gel. Western blot for $\beta_2$adaptin was performed to view potential interactions. Ponceau S staining of the GST proteins was done prior to western blotting to monitor levels of GST-fusion proteins used.

Connecting text

The following section (**Chapter 4**) entitled, "Biased Signalling by angiotensin analogs leads to differential βarrestin-dependent cellular responses" examines the role of the other major clathrin adaptor protein that is critical in receptor endocytosis, βarrestin. In **Chapter 2**, we used a biased ligand of the AT1R to demonstrate the βarrestin-dependency of a cellular event. Here, we attempt to characterize new βarrestin-biased ligands for the AT1R with the aim of understanding how signalling can affect an endocytic adaptor and vice-versa. While AT1R biased signalling via the compound SII has been done, SII represents a poor biased ligand for the AT1R maintaining at maximum 50% of the βarrestin-dependent signalling seen with AngII treatment. Furthermore, it has been shown that different ligands can mediate differential βarrestin conformations once recruited to the receptor but no functional significance was ever attributed to the these modifications. As such, the main aims of our study were as follows:

1) Characterize four different AngII analogs that we hypothesized would display biased signalling towards the βarrestin pathway in terms of their ability to activate G protein- and βarrestin-dependent signalling,

2) Establish whether receptor-mediated signalling can alter βarrestin functionally,

3) And establish a theoretical functional significance of the different βarrestin conformations that are possible through ligand variation,

Chapter 4

Differential βarrestin-dependent conformational signalling and cellular responses revealed by angiotensin analogs

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Abstract

The angiotensin type 1 receptor (AT1R) and its octapeptide ligand, angiotensin II (AngII) engages multiple downstream signaling pathways. Ligand-dependent AT1R functional selectivity has unveiled G protein- and βarrestin-dependent signaling. Here, we examine AT1R-mediated $G\alpha_q$ and βarrestin signaling using multiple AngII analogs bearing substitutions at the critical position Phe^8. Using assays that discriminate between ligand-promoted recruitment of βarrestin to the AT1R and its resulting conformational rearrangement and downstream signaling events, we extend the concept of biased signaling to include the analogs propensity to differentially promote βarrestin conformational changes. The relative contributions of GRK2 and GRK6 were also found to vary among analogs indicating a role for the kinases in these βarrestin responses. We demonstrate that the efficacy of AngII analogs at activating ERK1/2 correlated with the stability of the complexes between βarrestin and AT1R in endosomes, and not with the extent of βarrestin recruitment to the receptor, supporting the importance of the nature of this complex towards selective signaling modes. In vascular smooth muscle cells, we establish a link between βarrestin's conformational change promoted by ligands and their effect on βarrestin-dependent migration or proliferation responses. Our data reveal the existence of novel modalities of biased signaling at the AT1R and βarrestin level, and suggest that different AngII analogs selectively engage distinct βarrestin conformations leading to specific signaling events and cell responses.

1. Introduction

Angiotensin II (AngII) signaling primarily through the angiotensin type I receptor (AT1R) is implicated in various cardiovascular and renal disorders including hypertension, cardiac hypertrophy, myocardial infarction, congestive heart failure and nephrotic syndrome (1-3). The AT1R, a member of the G protein-coupled receptor (GPCR) family, couples to numerous signaling pathways including G protein- ($G\alpha_q$, $G\alpha_i$, $G\alpha_{12/13}$) and βarrestin-dependent signaling as well as receptor tyrosine kinase transactivation (4). G protein-coupled receptor kinase (GRK)-mediated receptor phosphorylation, increases the receptor's avidity for βarrestin (5) which leads to functional uncoupling of G protein signaling. While receptor-mediated G protein activation can lead to mitogen activated protein kinase (MAPK) signaling, initiating βarrestin complex formation with the receptor can also engage MAPK (6). GPCR-mediated transactivation of receptor tyrosine kinases has been shown to occur via both G protein- (7) and βarrestin-dependent (8) mechanisms. Multiple studies now point to the engagement of distinct signaling pathways of a given GPCR by different ligands; a phenomenon referred to as ligand-directed signaling. This involves the differential stimulation of G protein-dependent and βarrestin-dependent, G protein-independent signaling. For instance, biased peptide ligands stimulating only $G\alpha_s$-PKA-mediated ERK1/2 activation or G protein-independent, βarrestin-dependent ERK1/2 activation have been developed for the type 1 parathyroid hormone-related-protein receptor (PTH1R) (9). There are also numerous studies highlighting ligand-directed signaling for the β-adrenergic receptors (10-15).

For the octapeptide AngII, residues Tyr^4 and Phe^8 are essential for both AT1R ligand binding and G-protein-dependent signaling (16-18). Substituting position 4 and/or 8 in AngII such as seen with the widely used G protein-signaling antagonist [Sar^1, Ile^4, Ile^8]-Ang (SII) (where Sar is N-methylglycine) and the more recently reported AngII analog, TRV120027 ([Sar^1,D-Ala^8-OH]-AngII) (19, 20), produces βarrestin-selective signaling ligands. A large body of data now demonstrates SII-mediated βarrestin-dependent signaling downstream of the AT1R (21-23). Moreover, βarrestin biased ligands for AT1R have recently been demonstrated, both *in vitro* and *in vivo*, to have beneficial cardiac effects in animal models (20, 24, 25), suggesting that such biased

ligands' effects are likely dependent on the pluridimensionality of their efficacies towards distinct and/or common sets of signaling modalities, in particular the βarrestin-dependent ones. Given our limited knowledge on how AT1R-selective biased ligands function, understanding the structure-signaling relationship for AngII analogs and the specific molecular mechanisms underlying their biased activities is pivotal to improve their efficacies.

Here, using different AngII analogs we show that differential conformations of βarrestin promoted by these ligand-bound AT1R complexes impact the signaling capabilities of βarrestin. More importantly, we demonstrate that these analogs, which selectively, but differentially activate βarrestin-dependent signaling, have distinct functional outcomes.

2. Results

As a prelude to characterizing the signaling pathways promoted by AngII and its analogs ([Sar^1,Ile^8]-AngII (SI); [Sar^1,Ile^4,Ile^8]-AngII (SII); [Sar^1,Val^5,D-Phe^8]-AngII (SVdF); [Sar^1,Bpa^8]-AngII (SBpA) (where Bpa is benzyol-phenylalanine) and [Asp^1,Val^5,Gly^8]-AngII (DVG)) (Fig. 1A), we first assessed the affinities of each ligand for the HA-tagged AT1R stably expressed in HEK293 cells. The K_i of unlabelled AngII for displacing ^{125}I-AngII was found to be in the low nM range (~1 nM, Fig. 1B), whereas all tested analogs had lower affinities with K_i ranging from the low to medium nM range for SI, SVdF, SBpA and DVG (~7-18nM), to the higher nM range for SII (~213nM). We next examined receptor-mediated signaling events promoted by AngII and its analogs. AT1R-mediated inositol phosphate (IP_1) production was assessed, as well as PKCβ1-GFP activation and βarrestin-mRFP translocation were simultaneously monitored in living cells using confocal microscopy. AngII (1 μM) and its analogs (10μM) were used at concentrations occupying >95% of receptors. As expected, AngII induced a rapid and sustained recruitment of PKC to the plasma membrane (Fig. 2). Of the five analogs, SBpA and to a much lesser extent, SVdF induced PKC recruitment. Consistent with the PKC activation assay, we found that SBpA induced significant increases in IP_1 accumulation above baseline—around 28% of that of AngII (Fig. S1A and Table S1) —although with a potency 18 times lower than AngII. SVdF was as potent as SBpA to induced IP_1 production, but promoted an increase of 4% of the amounts seen with AngII. SI, SII and DVG promoted less than 1% (0.6%, 0.5% and 1%, respectively) of the inositol accumulation stimulated by AngII. In contrast to the activation of PLC (through IP_1 production) and PKC that were observed only for AngII, SBpA and SVdF, all AngII analogs promoted the formation of complexes between AT1R and βarrestin2 in endosomes, which were prominent at 15 min post-treatment, although to varying degrees (Fig. 2 and S1B). Immunoprecipitation experiments confirmed that βarrestin was recruited to the AT1R following treatment with AngII and any of the analogs (Fig. S1C).

To characterize both the potency and efficacy of βarrestin-recruitment to the AT1R, we employed a bioluminescence resonance energy transfer (BRET) approach that monitors the recruitment of βarrestin1 to the AT1R in living cells, following ligand stimulation. AngII is both the most potent and efficacious at promoting the recruitment

of βarrestin (Fig. S2A). SI, SVdF and SBpA recruited βarrestin with similar potency and efficacy to each other, while DVG was less potent but equally efficacious to these three analogs. SII displayed the lowest potency and efficacy of all ligands. Since recruitment of βarrestin to receptors leads to its conformational rearrangement that can be monitored using a BRET-based double-brilliance βarrestin biosensor (26), we next assessed whether the different AngII analogs could differentially modulate such changes in conformation. For this purpose, we monitored ligand-induced BRET changes using a new, optimized double-brilliance βarrestin1, where βarrestin is introduced between GFP[10] and RLucII. As BRET depends on the distance and the orientation between the RlucII and GFP10, changes in the signal would reflect a modification in the structure of βarrestin resulting in an alteration in the relative position of the enzyme and the fluorophore. Results show that the analogs have a wide array of potencies and efficacies in promoting changes in βarrestin conformation with AngII again being the most potent, while SI, SVdF, SBpA and DVG had similar efficacies but showed lower potencies (Fig. S2B). SII had the lowest potency and efficacy. Since GRK2- and GRK6-mediated phosphorylation have been shown to play differential roles in βarrestin recruitment to AT1R (27), and thus could play important roles in the conformational rearrangement of βarrestin following receptor binding, we next assessed the effect of selectively depleting GRK2 and GRK6 on both responses. Analysis of dose-response BRET curves for βarrestin recruitment to AT1R and conformational rearrangement of βarrestin in the presence and absence of siRNA selectively targeting the individual kinases (Fig. S3) reveals that depleting GRK2 had relatively little effect on AngII and all analogs on promoting these responses (Fig. 3, A-D). In contrast, GRK6 depletion led to readily detectable but distinct changes in both recruitment and conformational responses for many ligands (Fig. 3, E-F). To better appreciate these changes, a Cartesian plot of the GRK knockdown-promoted changes in both compounds' efficacy and potency to illicit βarrestin recruitment and conformational changes is represented in Fig. S4. This representation confirms that depletion of GRK2 led to modest increases (~20%) in AngII, SVdF, DVG and SII efficacies to promote βarrestin conformational rearrangement (Fig. S4, A-B). The potencies of the compounds in either the recruitment or the conformation assay were only modestly affected by the GRK2 depletion (less than 0.2 log units). When considering the effect of GRK6 on the βarrestin recruitment (Fig. S4C), no change in

efficacies and only modest reductions (less than 0.2 log units) in the potencies of AngII, SBpA and SVdF were observed. In contrast, both the potencies and efficacies of SI and DVG were diminished whereas for SII, the efficacy but not the potency was affected. With the exception of SBpA, the effects observed in the recruitment assays were amplified for conformational rearrangement (Fig. S4D) as illustrated by a further reduction in the compounds potency and/or efficacies, which are particularly evident for SI and SII. In addition to showing that GRK6 has a greater influence than GRK2 on both βarrestin recruitment and conformational rearrangement; our data clearly indicate that the βarrestin responses promoted by distinct ligands are differentially affected by the GRK knockdown.

Collectively, our data show that some AngII analogs differentially regulate AT1R-mediated responses in distinct ways. To quantitatively assess the biases of the ligands toward the different responses, we used the recently described operational model method (15, 28, 29). This method allows one to calculate the analogs' efficacy relative to their affinities for engaging a specific signaling pathway (*i.e.* σ factor, which corresponds to the signaling efficiency of the pathway), and to determine if this pathway is favoured over another (*i.e.* bias factor, β) as compared to AngII, which is set to be balanced for both signaling events. The operational model also allows for the comparison of pathways that may be subjected to differential amplification (*e.g.* G protein vs. βarrestin). Analysis of the G protein-dependent response as assessed by IP_1 generation vs. recruitment of βarrestin reveals a significant bias of SVdF and SBpA towards the latter response (Table 1). The weak IP_1 signals (Fig. S1A and Table S1) generated by SI, SII and DVG preclude from calculating a formal bias factor, inferring a *de facto* bias of these analogs toward a βarrestin response. We also analyzed the bias of the ligands to promote βarrestin conformational rearrangement vs. recruitment to AT1R. In control cells, results show that SI, SII and SBpA were significantly biased towards βarrestin recruitment, while SVdF and DVG were not (*i.e.* more balanced towards each response) (Table 2 and S2). Thus, for the same level of recruitment, SI, SII and SBpA are less efficient at promoting a conformational rearrangement in βarrestin as compared to AngII. GRK2 depletion led to all analogs being biased towards recruitment except for SBpA, which became balanced at both responses. For GRK6 knockdown, only SI and SII

remained significantly biased towards recruitment. In fact, the bias factor for SII became even greater following GRK6 depletion, consistent with the notion that this GRK plays an important role for the SII-promoted βarrestin conformational rearrangement (Fig. S4). To better appreciate how GRK depletion relatively affected the efficiency (as defined by the σ factor) of the analogs to promote each βarrestin response, we plotted the σ factor of βarrestin conformational change as a function of its recruitment's σ factor (Fig. 4 and Table S2). This analysis showed that GRK2 depletion had only minor effects on both responses induced by all analogs (Fig. 4A and 4B). On the other hand, the efficiency of both signaling responses induced by SI and DVG were markedly decreased in GRK6 depleted cells (Fig. 4C). SBpA became more efficient at inducing βarrestin conformational rearrangement making it more balanced, which contrasted with the decreased efficiency at this response seen with SII. Little change in signaling efficiency of either pathway was seen with SVdF in GRK6 depleted cells.

Given the differences observed in βarrestin recruitment and conformational rearrangements among the AngII analogs, we wondered how the complex between βarrestin and the receptor in endosomes were affected by the different ligands. We took advantage of a fluorescence recovery after photobleaching (FRAP) approach, a technique we recently described, which allows monitoring the avidity between βarrestin and receptors in live cells (30, 31). This avidity is inversely proportional to the recovery rate since the exchange of a bleached-, receptor-bound βarrestin on the endosome by an unbleached free one is limited by the strength of the former complex. We observed that the βarrestin recovery rate on endosomes varied with the ligands, AngII and DVG being at opposite ends of the spectrum (Fig. 5A). βarrestin recovery on AT1R containing endosomes was significantly faster with DVG than with AngII, indicating that the former ligand-promoted interaction between βarrestin and AT1R was more labile than the one induced by AngII. Linear regression analysis of the recovery curves (Fig. 5B) enabled the determination of recovery half-times on endosomes. Following AngII treatment, 2.2 min are required for 50% recovery of the fluorescence, whereas the analogs displayed faster recovery times with half-lives ranging from 0.8 to 1.7 min (Fig. 5C). βarrestin-containing endosomes induced by SII were very small and motile making it difficult to quantitatively assess βarrestin recovery (Fig. S1B). Nonetheless, we could qualitatively appreciate from

SII treated cells that the fluorescence recovered rapidly post-bleaching of the endosomes.

Since it represents a point of convergence downstream of many AT1R-mediated signaling pathways (*e.g.* G-protein and βarrestin-dependent), we next examined the abilities of AngII and the analogs to promote AT1R-mediated ERK1/2 activation. AngII robustly activated ERK1/2 in both a rapid and sustained manner, whereas the analogs did so with lower potencies and efficacies (Fig. 6A and Fig. S5), revealing the analogs as partial agonists at this pathway. Interestingly, the three ligands, AngII, SBpA and SVdF, which activated both the $G\alpha_q$ protein-dependent (IP_1 production and PKC activation) and βarrestin-dependant pathways, led to the greater ERK1/2 responses. To determine if this ERK1/2 activation was βarrestin-dependent, shRNA targeting βarrestin1 and βarrestin2 were used. Fig. 6B illustrates that after βarrestin1 or 2 depletion, ERK1/2 activation was significantly reduced for each ligand indicating a contribution of βarrestin-mediated signaling in all cases. Depleting βarrestin1 and βarrestin2, had the most effect on SI-, SII- and DVG-mediated ERK1/2 activation, consistent with the primary involvement of βarrestins in that response. Since βarrestin is involved in endosomal MAPK signaling (32), we examined the relationship between ERK1/2 activation and the avidity of complex between βarrestin and AT1R. As shown in Fig. 6C, we found a strikingly linear relationship between the degree of interaction of βarrestin with AT1R induced by the different AngII analogs and the activity of ERK1/2 at 15 min post agonist treatment (R^2=0.8662, $p<0.05$). Importantly, we found no correlation between the efficacy of ERK1/2 activation and the affinity of the ligands for the receptor, their efficacy to recruit or alter βarrestin's conformation (Fig. S6, A-C). Taken together these results indicate that, although all analogs tested (including some that cannot activate $G\alpha_q$) can promote βarrestin recruitment, their ability to induce conformational rearrangements and high avidity complex with the receptor vary greatly among ligands. Among all parameters assessed, the ability to promote stable complexes between AT1R and βarrestin represents the best predictor of MAPK activation response.

Finally, we wanted to study the consequences of ligand-mediated functional selectivity in a physiologically relevant cell line, rat aortic vascular smooth cells (VSMCs). We first

assessed the ability of AngII and analogs to induce cell migration, a well-characterized AT1R-dependent event (33). Treatment of cells with AngII led to a doubling in cell migration, which was significantly decreased by knockdown of βarrestin 1 or βarrestin 2 (Fig. 7A). Interestingly, all the analogs except DVG were able to recapitulate the migration and its βarrestin dependency, which mainly required βarrestin2, as revealed by knockdown using siRNA (Fig. 7A). Given the apparent uniqueness of DVG, we sought to further monitor the conformation status of βarrestin following treatment with DVG compared to AngII. Using a FRET-based intramolecular βarrestin2 sensor, we determined that while stimulation with AngII led to a significant increase in FRET signal, DVG treatment produced a significant decrease, suggesting ligand-mediated conformational differences in βarrestin (Fig. 7B). AngII has also been shown to induce VSMC growth (reviewed in (34)), therefore we examined the analogs' ability to induce this response. Using a ^{3}H-thymidine incorporation assay to measure cell proliferation, we found that while AngII leads to cell growth, of all the analogs only DVG mimicked this effect (Fig. 7C). As was the case for the migration response, AngII- and DVG- mediated cell growth was only blocked by βarrestin2 knock-down (Fig. 7D).

3. Discussion

The data presented here underscores the multifaceted regulation of βarrestin's change in conformation and directed signaling by providing new evidence that distinct complexes between βarrestin and AT1R promoted by AngII analogs lead to different βarrestin activated states and cellular outcomes. Furthermore, we reveal that ligands have different propensities to induce βarrestin conformational rearrangement upon recruitment to the receptor most likely as a result of distinct receptor conformations being promoted or stabilized by the analogs and selective GRK actions. We also show that the stability of the complex between βarrestin and AT1R in endosomes depends on the nature of the ligand-bound receptor complex and that this parameter correlates well with the degree of MAPK activation induced by the ligands. Our study further unveils novel high affinity biased βarrestin AngII ligands, which are able to engage selective subsets of AT1R-mediated responses (*i.e.* cell growth vs. migration). Our findings clearly validate the notion that distinct βarrestin-dependent signaling events can be selectively induced, and that functional segregation of cell responses can be achieved by the use of specific AngII analogs.

Previous studies have shown that a number of AT1R ligands are biased towards βarrestin vs. $G\alpha_q$ signaling (20, 21, 24). Here, we add to the list of such biased ligands by characterizing four AngII analogs that all favor the βarrestin response over the production of IP_1 and PKC activation. In some cases, the analogs behaved as strong partial agonists for the arrestin pathway while being essentially unable to promote $G\alpha_q$ signaling (SI and DVG). More importantly, our study revealed an additional ligand biased signaling modalities for βarrestin level by monitoring both the ligand-mediated interaction between AT1R and βarrestin and the ensuing conformational rearrangements of βarrestin using two distinct BRET-based assays. Although, it is still unclear to what extent these changes in βarrestin's conformation are structurally similar (*i.e.* whether all compounds promote a similar conformational rearrangement of βarrestin upon recruitment to the ligand-bound receptor), we nonetheless observed marked differences both in the recruitment and conformational rearrangement of βarrestin among the ligands. For some ligands there was a good congruence between the extent of βarrestin's conformational rearrangement and its recruitment to the

receptor (Table 2). However, for other ligands we observed a clear disconnect between the recruitment and the conformational change. For instance, for SI, SII and SBpA, there was less βarrestin conformational change for an equivalent degree of recruitment to the receptor resulting in a clear bias in the efficiency towards βarrestin recruitment (Fig. 4A and table 2). Furthermore, the observation that DVG promoted conformational changes distinct from that of AngII using the FRET-based sensor opens the possibility for ligand-specific βarrestin conformations. Together these results imply that the conformational change of βarrestin promoted by the ligand is dependent on its recruitment and binding to the receptor but that other receptor-dependent events that may be differentially triggered by distinct ligands can also contribute to the structural change in βarrestin.

The propensity of different analogs to promote distinct βarrestin recruitment and conformational rearrangement could in part be due to distinct GRK-mediated phosphorylation events triggered by the different ligands. Indeed, it was previously shown that ligand promoted phosphorylation of distinct sites by different GRKs, differentially affect the conformation of βarrestin following the stimulation of the β_2-adrenergic receptor (35). Our findings on the role of GRK2 and GRK6 in the efficiency (σ factor) of βarrestin conformational rearrangement and recruitment to AT1R further imply distinct functions of the kinases in these responses. Overall, GRK2 depletion had only relatively modest effects on either recruitment or conformational changes whereas GRK6 had much more noticeable effects on both the potencies and efficacies of various ligands to promote the two responses. Our findings that the efficacy of recruitment (*i.e.* Emax) of βarrestin to AT1R by AngII or SII were more affected by GRK6 than GRK2 depletion are in agreement with a recent report (24). However, they differ as our data also underscore the importance of GRK6 in regulating both the potency and efficacy of SI-, SII, and DVG-mediated βarrestin conformational changes. The fact that GRK2 depletion had little effect suggests either other kinases play redundant roles or GRK2 is not required for these responses in the cells studied. For the effects of GRK6 depletion, compounds can be divided in two groups: 1) modestly affected compounds (AngII, SBpA and SVdF) and 2) compounds whose propensity to promote βarrestin recruitment and conformational rearrangement was considerably reduced (SI, SII and DVG). Interestingly, the relative effect of GRK6 depletion on the efficacies and potencies of SI, SII and DVG

differed among the ligands as a function of the assay considered indicating different roles of the kinase in promoting the recruitment and stabilizing discrete conformations of βarrestin upon binding of different ligands. For example, the observation that GRK6 depletion had a particularly important effect on the potency of SII to elicit a conformational rearrangement compared to its effect on the recruitment suggests an important role for the kinase in promoting the structural change of βarrestin following its recruitment to the receptor. This important effect on the βarrestin conformation resulted in a significant increase in the bias factor for this ligand in the GRK6-depleted cells (Table 2). This may indicate a greater propensity of the SII-bound AT1R to act as a substrate for GRK6 and/or a greater GRK6 dependency of the SII-bound AT1R (e.g. via receptor phosphorylation) to promote a βarrestin conformational rearrangement. The present results clearly indicate GRK6 as an important regulator of βarrestin engagement by the AT1R that may contribute to the ligand biased signaling observed for this receptor.

In addition to the different propensity of the ligands to promote the engagement and the conformational rearrangement of βarrestin, the ligands were also found to be different in their ability to promote a stable receptor-βarrestin complex. We found a strong relationship between the stability of the complex between βarrestin and AT1R in endosomes promoted by AngII analogs and their degree of ERK1/2 activation. In contrast, the extent of recruitment and the conformational changes imparted on βarrestin by the ligands, do not correlate with their effectiveness at ERK1/2 activation (Fig. S6B and S6C). These results demonstrate that factors affecting the strength of the receptor and βarrestin complex are more likely to impact ERK1/2 signaling rather than the ones altering the amount of βarrestin recruitment to the receptor. Longer lived complexes between AT1R and βarrestin in the endosomes may enable the formation of more stable MAPK scaffold (*e.g.* with MEK and Raf) at this complex, thus allowing more efficient signaling. While the use of biased ligands favoring such signaling modes should help better appreciate the biological and physiological importance of receptor-mediated, βarrestin-dependent ERK1/2 signaling in endosomes, more work is needed to understand the underlying mechanisms regulating such complex formation in this cellular compartment.

Our data provide further evidence for the role of amino acid 8 of AngII in AT1R functional signaling selectivity (36, 37). We show that the aromatic and bulky nature of Phe^8 of AngII contributes to maintain G protein-dependent signaling, as SVdF and SBpA still retained some $G\alpha_q$-PLC-PKC-mediated signaling activity in HEK293 cells. Replacing Phe^8 in AngII with smaller non-aromatic residues (*e.g.* Ile and Gly in SI, SII and DVG) generated analogs lacking G protein-dependent signaling capabilities, consistent with what was previously reported (37). Interestingly, such substitution produced AT1R ligands with abilities to selectively promote functionally distinct conformations in βarrestin, affecting the stability of the signaling complex generated by the association of AT1R and βarrestin, and the cellular responses. Our results entail that SI and SII, through AT1R binding, are more selective for engaging βarrestin-dependent signaling than SVdF and SBpA, which also promoted G protein signaling. DVG demonstrated negligible amounts of ERK1/2 activation and $G\alpha_q$ activation despite being efficient in the recruitment of βarrestin to AT1R and promoting changes in βarrestin's conformation. Surprisingly, this analog was as potent as AngII to promote βarrestin-dependent cell growth in VSMCs, yet unable to induce migration. In contrast, all the other AngII analogs were as effective as AngII to promote βarrestin-dependent migration, yet lacked the ability to potentiate cell growth. Given the FRET data in VSMCs demonstrating that AngII induced a different conformation than DVG, these results suggest that βarrestin biased signaling depend on the selective conformation of βarrestin imposed by the ligand-receptor complex. DVG would thus allow βarrestin-dependent selective engagement of adaptors and/or signaling effectors common to AngII for VSMC growth, but would not allow the engagement of effectors involved in migration. Resolving these βarrestin-mediated conformationally-dependent signaling structures and understanding their underlying mechanisms of regulation, remains an important challenge.

While the results presented here increase the complexity of the landscape of biased signaling by highlighting a βarrestin conformationally-dependent mode of regulation, it also yields the potential to connect specific signaling pathways with their

physiological outputs. Use of such AngII biased ligands should not only help further our understanding on the role of βarrestin-mediated signaling in normal and pathophysiological conditions, but could eventually be exploited for therapeutic purposes.

4. Materials and methods

Materials

Angiotensin II, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), collagen (type I/calf) and poly-l-lysine are from Sigma Chemical Co. Sar^1,Ile^8-AngII (SI) is from MP Biomedicals. Sar^1,Ile^4,Ile^8-AngII (SII), Sar^1,Val^5,D-Phe^8-AngII (SVdF), Sar^1,Bpa^8-AngII (SBpA) and Asp^1,Val^5,Gly^8-AngII (DVG) were synthesized by us. DMSO was purchased from Bioshop. Antibodies against pERK1/2, total ERK1/2 and GRK2 were from Cell Signalling Technology. The IP-One HTRF® assay was from CisBio (Bedford, MA). Anti-phosphotyrosine (4G10) was from Millipore. The polyclonal antibody against the C-terminal domain of βarrestins (3978) was described elsewhere (38). Anti-HA antibodies coupled to agarose beads and mouse anti-HA (clone 12CA5) were purchased from Roche. GRK6 antibody was from Santa Cruz. Anti-βadaptin antibody was from BD Biosciences. Anti-mouse and anti-rabbit HRP-conjugated IgG were from Sigma. Western Lightning® Plus–ECL, ^{3}H-thymidine and ^{125}-Iodine were obtained from Perkin-Elmer. Scrambled siRNA and siRNA against rat βarrestin1 (rUrGrCrCrArCrArGrUrArUrCrArUrCrArGrCrUrUrCrCrUrCrCrArU and rGrCrUrCrUrArUrGrArGrArUrUrGrGrUrGrUrCrUrArCrUrGrGrArG), and βarrestin2 (rGrCrUrCrArGrGrArArArCrGrArArGrArUrArUrGrGrCrCrUTG, rGrGrArGrUrArGrArCrUrUrUrGrArGrArUrUrCrGrArGrCCT and rArGrArCrCrGrUrCrArArGrArArGrArUrCrArGrArGrUrGTC) were purchased from IDT. Control siRNAs and siRNAs against human GRK2 and GRK6 were purchased from Dharmacon, being respectively siGENOME Non-Targeting siRNA pool#1 (catalog number D-001206-13-05), siGENOME SMARTpool human ADRBK1 (catalog number M-004325-02-0010) and siGENOME SMARTpool human GRK6 (catalog number M-004627-02-0010). Boyden chambers (Corning), pre-coated iodination tubes (Thermo Scientific) and acetonitrile were purchased for Fisher Scientific. Sep-Pak® Plus C18 cartridges are from Waters.

Plasmids and constructs

Plasmids encoding HA-AT1R, PKCβ1-GFP, βarr2-YFP, βarr2-mRFP were described elsewhere (38-40). The GFP^{10}–βarr1–RlucII and βarr1–RlucII constructs were derived

from previously published GFP^{10}-EPAC-RlucII fusion protein (41) by excising the EPAC coding sequence with Acc65I-HindIII and NheI-HindIII restriction enzymes respectively, and replacing it with a PCR-amplified coding sequence of human βarrestin1. The CFP-βarr2-Venus was generated from a CFP-EPAC-Venus fusion protein derived from a previously published CFP-EPAC-RlucII construct (42) by replacing EPAC by PCR amplified human βarrestin2 between Acc65I and HindIII restriction sites. All constructs were verified by DNA sequencing (Sequencing Service, Genome Quebec Innovation Centre, McGill University, Quebec, Canada)

Cell culture and transfection

HEK293 cells stably expressing the AT1R (293HA-AT1R) were grown in MEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco) and 100 µg/mL gentamicin (Gibco). Cells were seeded in six well plates ($1.5x10^5$ cells/well), 35 mm glass-bottomed culture dishes (Mattek Corp.) ($1.2x10^5$ cells/dish) or 10 cm dishes ($1.5x10^6$ cells/dish). Transfections were performed by a calcium phosphate co-precipitation method as previously described (43). Rat aortic vascular smooth muscle cells (VSMCs) were a gift from Dr. M. Servant (Université de Montréal). They were grown in low-glucose DMEM (Hyclone) supplemented with 10% fetal calf serum (FCS, Gibco), 2mM L-glutamine and 100µg/ml gentamicin. Cells were seeded in 24 well plates at a density of $1.0x10^4$ cells/well (^{3}H-thymidine experiments), $8.8x10^5$ cells/10cm dish (IP1 experiments) and $1.5x10^4$cells/well (96 well plate) for AlphaScreen experiments. For experiments with siRNA, cells were transfected by lipofectamine 2000 (Invitrogen) with 20nM siRNA (control or anti-βarrestin) in serum free medium. 4h later, an equal amount of media containing 20% FCS was added, to return the cells to 10% FCS overnight. The next day, cells were split into 24 well plates (10^4 cells/well), allowed to adhere for 4h and then serum starved for 48 hours, at which point the experiment was performed.

Labeling of tracer and affinity determination

^{125}I-AngII was prepared using Iodogen and its specific radioactivity (1051 ci/mmol) was determined from self-displacement and saturation experiments as previously described (44). Dose-displacement experiments to determine the IC_{50} for the

analogs was performed with slight modifications to a published protocol (44). Briefly, 293HA-AT1R cells were grown in 10cm dishes to confluence. Cells were washed with PBS, and then resuspended in PBS containing 2mM EDTA. Cells were dosed using the Lowry-HS assay (Biorad), so that 25-50µg could be added per reaction. Cells were resuspended in 0.5 ml binding buffer (2% BSA, 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1mM PMSF, 25µg/ml leupeptin, 2.5µg/ml aprotinin, and 1mM pepstatin). Approximately 90,000cpm of ^{125}I-AngII was added to each tube (~0.11 nM) and was displaced with varying concentrations of cold ligands for 1h at room temperature. Bound radioactivity was separated from free ligand by filtration through GF/C filters pre-soaked in binding buffer. The radioligand content was evaluated by γ-counting on a Perkin Elmer Wizard 1470 automatic gamma counter. The B_{max} (375 fmol/mg of total proteins) and K_d for ^{125}I-AngII (0.45 nM) was determined from saturation binding experiment using 293HA-AT1R cells. Apparent affinities (K_i) of AngII and its analogs were calculated from the IC_{50} from radioligand displacement curves using the equation: $K_i = IC_{50}/(1+[ligand]/ K_d)$.

IP1 assay

The assay was performed to manufacturer's specifications. Briefly, 10^4 cells/well (384 well-plate) were treated with increasing concentration of AngII or AngII analog for 30 min. IP1-d2 and anti-IP1-cryptate were added for 2 hours. Plates were read on a Synergy 2 multi-mode microplate reader.

ERK1/2 phosphorylation

293HA-AT1R cells were seeded in 6-well plates coated with poly-L-lysine. 48h post-seeding, cells were serum starved in MEM containing 20mM HEPES and then left untreated or treated with AngII (1µM) or analogs (10µM). Cells were lysed in Laemmli buffer (250 mM Tris–HCl pH 6.8, 2% SDS (wt/vol), 10% glycerol (vol/vol), 0.01% bromophenol blue (vol/vol), and 5% β-mercaptoethanol (vol/vol) and sonicated. Lysates were resolved on SDS-PAGE, transferred to nitrocellulose membranes and blotted for pERK1/2 and ERK1/2.

Alphascreen assay

ERK1/2 activation in 293HA-AT1R cells was monitored with Perkin Elmen's AlphaScreen SureFire assay as previously reported (45). Cells were seeded in 10cm dishes, transfected using lipofectamine 2000 with shRNA against βarrestin1 or βarrestin2 and 24h later, reseeded in 96 well plates (10^4 cells). For experiment without transfection, cells were directly seeded in 96 well plates (10^4 cells). 48h later, cells were serum-starved overnight. Cells were then stimulated for 5 or 15min with 1μM AngII or 10μM AngII analog, and lysed in 30μl of lysis buffer. Cells were incubated at RT on a plate shaker for 10 min. 5μl of lysate was used for analysis. Readings were performed on a Perkin Elmer Enspire 2300 multilabel reader.

Confocal microscopy

293HA-AT1R cells were plated in 35mm dishes glass bottom dishes and transfected with 1) βarrestin2-mRFP and PKCβ1-GFP or 2) βarrestin2-pEYFP. 48h post-transfection cells were serum starved, followed by stimulation with AngII (1μM) or analogs (10μM). For (1) PKC translocation and βarrestin endosome formation was monitored for 30 minutes. For (2), 15min after ligand addition, endosomes were bleached and fluorescence recovery was monitored over a period of 5 minutes, with photos taken every 30s. Images were collected on a Zeiss LSM-510 Meta laser scanning microscope with a 40x oil immersion lens using the following excitation/emission filter sets: 543nm/LP560nm for mRFP, 488nm/BP 505-530 for GFP and 514nm/BP530-600nm for YFP.

Immunoprecipiation experiments

Coimmunoprecipitation of covalently cross-linked βarrestin to AT1R was performed as previously described (40). Western blot was performed using the anti-βarrestin antibody (3978) and anti-HA antibody (12CA5).

Bioluminescence Resonance Energy Transfer (BRET) Assay

BRET experiments were performed as previously described (46, 47). Briefly, HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin (Wisent Inc). For experiments without siRNA, $3.0x10^6$ cells

were seeded in 10cm dishes. Transient transfection was performed 24h later using polyethyleneimine (PEI; Polysciences) at a DNA:PEI ratio of 1:3. 5µg of AT1R-YFP and 500ng of βArr1-RlucII, or 5µg of HA-AT1R and 200ng of GFP_{10}-βarr1-RlucII were co-transfected and the total amount of DNA transfected in each plate was adjusted to 10µg with salmon sperm DNA (Invitrogen). For experiments with siRNA, 1.0 x 10^7 cells were seeded in 10cm dishes in serum free medium 24h before transfection. Transient transfection was performed using lipofectamine 2000 (Invitrogen) with the same amounts of DNA than described above and with 20nM siRNA (Dharmacon). 24h post-transfection, cells were detached, seeded in pre-treated poly-L-ornithine hydrobromide (Sigma-Aldrich) 96-well white plates at 50,000 cells per well, and re-incubated at 37 °C for an additional 24h before being processed. Cells were washed once with Tyrode's buffer (140 mM NaCl, 1 mM$CaCl_2$, 2.7mM KCl, 0.49mM $MgCl_2$, 0.37mM NaH_2PO_4, 5.6mM glucose, 12mM $NaHCO_3$, 25mM HEPES, pH 7.5) directly in the 96-well plates and incubated in buffer with or without indicated concentrations of AngII and analogs for 25 min at room temperature. Coelenterazine-H (Nanolight Technology) or coelenterazine 400A (Biotium) was added to a final concentration of 5µM in Tyrode's buffer 5min before reading. Readings were collected using a MITHRAS LB940 multidetector plate reader (Berthold Technologies) allowing the sequential integration of the signals detected in the 480±20- and 530±20-nm windows for the RlucII *Renilla* luciferase and YFP light emissions, respectively, and in the 410±40- and 515±15-nm windows for the RlucII and GFP_{10} light emissions, respectively. The BRET signal was determined by calculating the ratio of the light intensity emitted by the YFP or GFP_{10} over the light intensity emitted by the RLucII. The values were corrected by subtracting the background BRET signals detected when RlucII was expressed alone.

Migration assay

This assay was previously described using HEK293 cells but was modified for VMSCs (48). Briefly, VSMCs were serum-starved overnight and seeded into Boyden chambers (24-well inserts with 8-µm pore collagen-coated membranes). One hour after plating, cells were stimulated with AngII (100 nM), AngII analog (10 µM) or left untreated. After 4 h, cells were fixed using paraformaldehyde (4%) for 20 min and incubated with crystal violet (0.1% in 20% MeOH), overnight. Membranes were

washed three times in dH_2O, and cells were removed from the upper chamber, leaving those that migrated through to the lower chamber. Cells were detached and counted.

Fluorescence Resonance Energy Transfer (FRET) Assay

Vascular smooth muscle cells were transfected using lipofectamine 2000 at a DNA:lipofectamine ratio of 1:3. AT1R-HA (2µg) and βarrestin2 (4µg) tagged at both the N- (CFP) and C- (Venus) termini were co-transfected. pcDNA3.1 zeo+ replaced βarrestin in the mock condition. 24 hours post transfection, cells were serum-starved overnight. Cells were detached the following morning in Tyrode's buffer and seeded into 96-well plates (50,000 cells/well) and incubated at 30°C. 4 baseline readings were taken on a Perkin Elmer Enspire 2300 multilabel reader with shaking between each read to prevent cells from settling. Cells were then treated with buffer alone, 100nM AngII or 10µM DVG and monitored for 16min with readings every minute. Values were corrected by subtracting the background signal from cells not expressing the βarrestin sensor.

^{3}H-thymidine incorporation assay

VSMCs were seeded in 24 well plates. 24h later, media was replaced with low-glucose DMEM containing 0.2% FCS. 48h later, cells were placed in serum free medium with either DMSO or AG1478 (125nM) and 0.5µCi of ^{3}H-thymidine (concentration of 1 µCi/ml). Cells were then treated with media alone, or media containing AngII (final concentration, 100nM) or analogs (final concentration, 10 µM). 24h later, cells were put on ice washed twice with cold PBS, followed by 30min incubation with 5% TFA. Finally, cells were washed with PBS, and radioactivity was recovered in 0.5ml 1M NaOH, which was added to vials containing 5ml of scintillation liquid and counted using Perkin Elmer Tri-carb 2800TR liquid scintillation analyzer.

Data Analysis

Intensity of the signals from Western blots was determined by densitometric analysis using ImageJ, and is presented as the mean ± SEM of at least three independent experiments. Bias factors (β) and sigma values (σ) were calculated using the operational model as described previously (15, 28). Statistical analysis was performed using Graphpad Prism software using one-way or two-way ANOVA when appropriate.

Bonferroni (comparison between all groups) or Dunnett's (comparison to control) post-hoc tests were performed where necessary.

Acknowledgements

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The abbreviations used are: GPCRs, G protein-coupled receptors; ERK1/2, extracellular signal-regulated protein kinases; AT1R, angiotensin II type 1 receptor; YFP, yellow fluorescent protein, PKC, protein kinase C; mRFP, monomeric red fluorescent protein, GRK, G protein-coupled receptor kinase, BRET, bioluminescence resonance energy transfer, FRET, fluorescence resonance energy transfer, VSMC, vascular smooth muscle cell, RLuc, *Renilla* luciferase, FRAP, fluorescence recovery after photobleaching, IP1, inositol-1-phosphate, Sar, N-methylglycine, Bpa, benzoyl phenylalanine.

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Figures and legends

A

AngII	Asp-Arg-Val-Tyr-Val-His-Pro-Phe
SI	Sar-Arg-Val-Tyr-**Ile**-His-Pro-**Ile**
SII	Sar-Arg-Val-**Ile**-Val-His-Pro-**Ile**
SVdF	Sar-Arg-Val-Tyr-Val-His-Pro-**D-Phe**
SBpA	Sar-Arg-Val-Tyr-Val-His-Pro-**Bpa**
DVG	Asp-Arg-Val-Tyr-Val-His-Pro-**Gly**

B

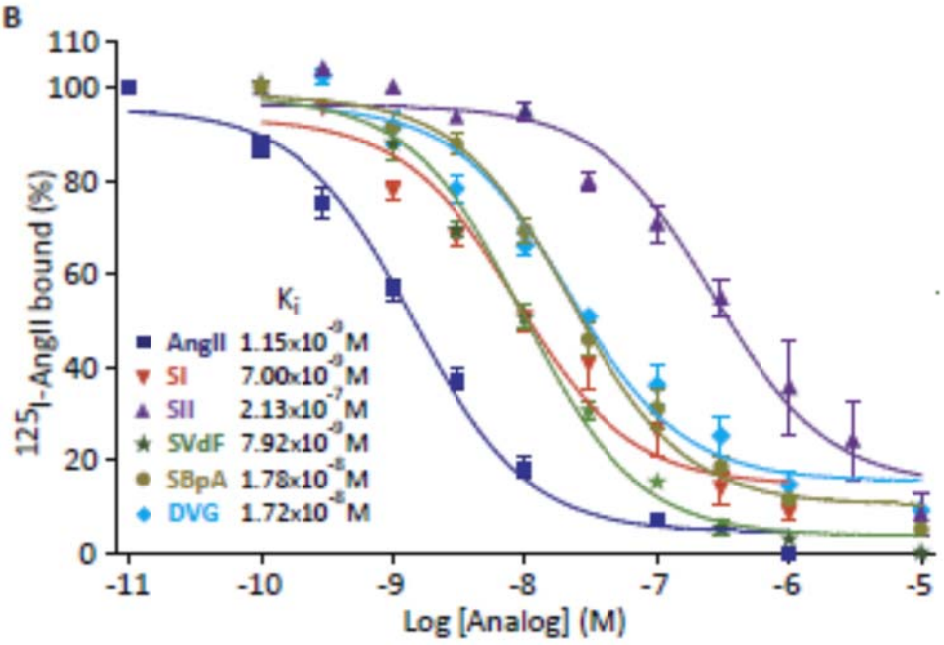

Fig. 1. Binding and affinities of AngII analogs at the AT1R

A, AngII ligands used in this study. Residues in bold are different from the AngII sequence. Sar and Bpa represent N-methylglycine and benzoyl-phenylalanine residues, respectively. B, Affinities of AngII analogs for AT1R. Non-linear regression, one-site competition curve were generated from competitive radioactive ligand binding assays with values normalized to percent based on specific binding. K_i values were calculated from the IC_{50} as described in the materials and methods. Average values for all compounds are represented by 6 independent experiments done in triplicate.

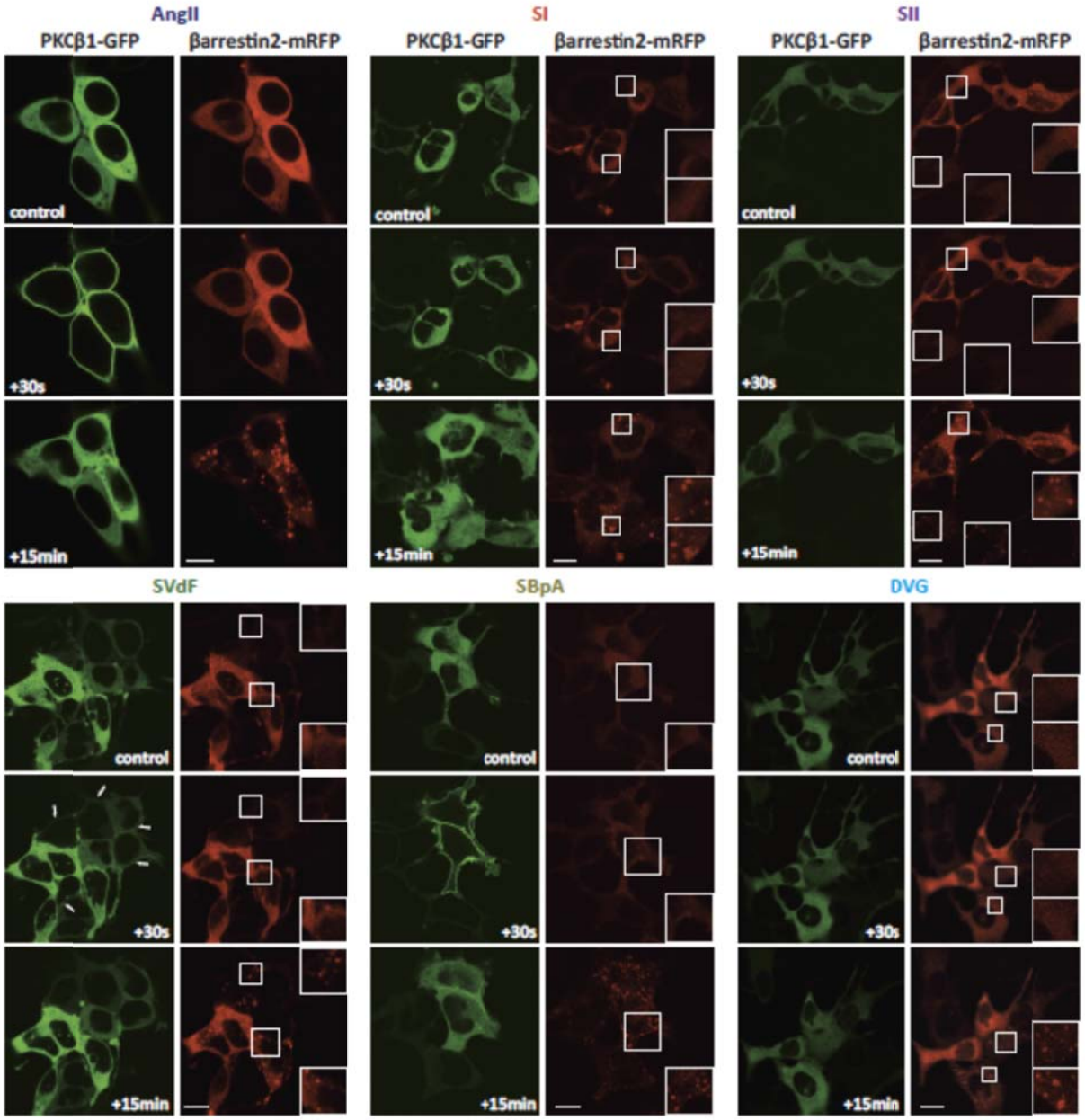

Fig. 2. G protein- and βarrestin-dependent signaling of AngII analogs at the AT1R. HA-AT1R cells transfected with PKCβ1-GFP and βarr2-mRFP were treated with either 1μM AngII or 10μM analog. Cells were monitored live and images were taken over 30 minutes. Images after 30s and 15min are shown. Images are representative of at least 5 cell clusters in three independent experiments. Scale bars represent 10μm. Insets are zooms of indicated regions of the cell.

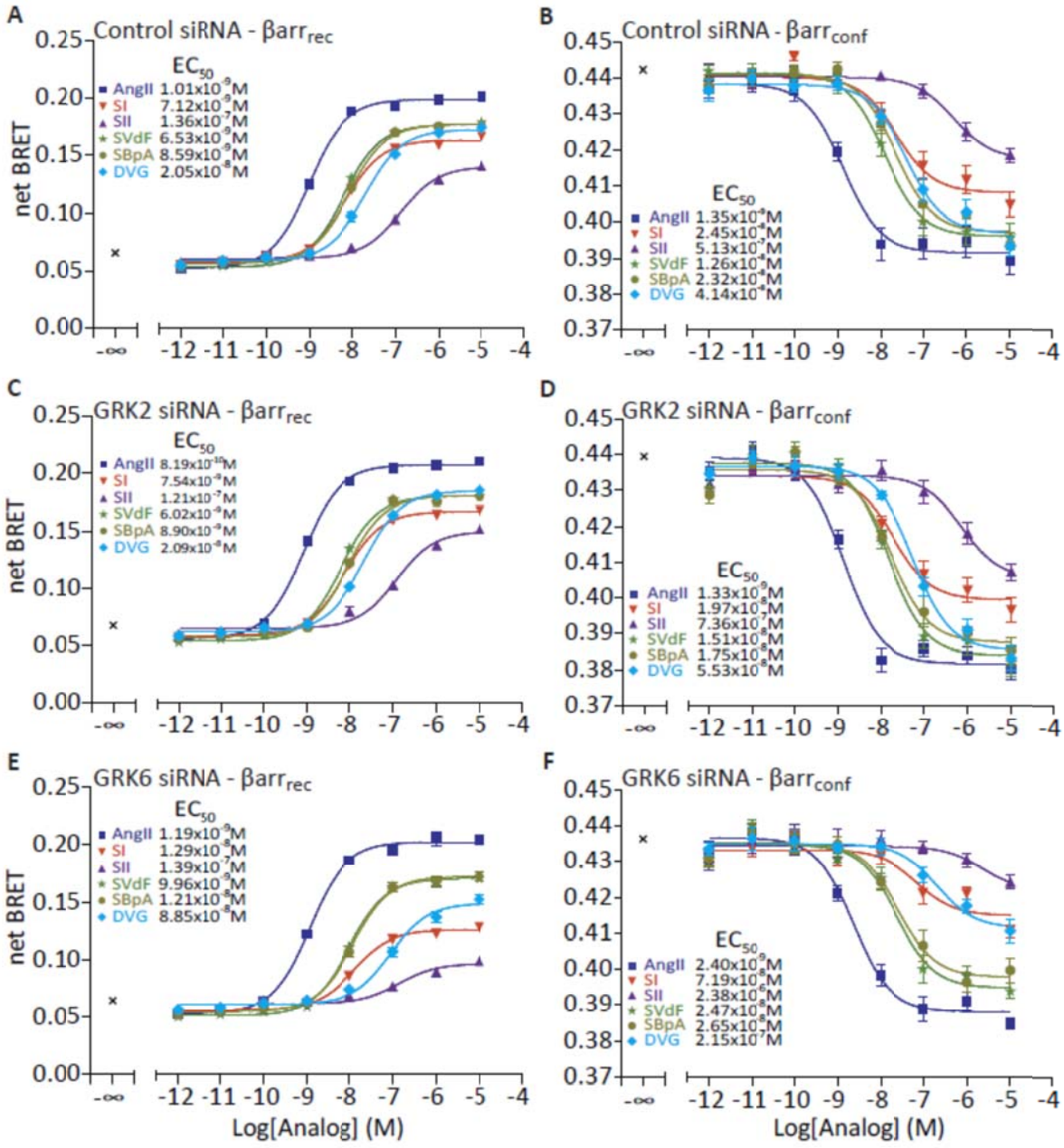
A
Control siRNA - βarr$_{rec}$
net BRET
EC$_{50}$
AngII 1.01x10^{-9}M
SI 7.12x10^{-9}M
SII 1.36x10^{-7}M
SVdF 6.53x10^{-9}M
SBpA 8.59x10^{-9}M
DVG 2.05x10^{-8}M
B
Control siRNA - βarr$_{conf}$
EC$_{50}$
AngII 1.35x10^{-4}M
SI 2.45x10^{-4}M
SII 5.13x10^{-7}M
SVdF 1.26x10^{-4}M
SBpA 2.32x10^{-4}M
DVG 4.14x10^{-4}M
C
GRK2 siRNA - βarr$_{rec}$
net BRET
EC$_{50}$
AngII 8.19x10^{-10}M
SI 7.54x10^{-9}M
SII 1.21x10^{-7}M
SVdF 6.02x10^{-9}M
SBpA 8.90x10^{-9}M
DVG 2.09x10^{-8}M
D
GRK2 siRNA - βarr$_{conf}$
EC$_{50}$
AngII 1.33x10^{-9}M
SI 1.97x10^{-6}M
SII 7.36x10^{-7}M
SVdF 1.51x10^{-8}M
SBpA 1.75x10^{-8}M
DVG 5.53x10^{-8}M
E
GRK6 siRNA - βarr$_{rec}$
net BRET
EC$_{50}$
AngII 1.19x10^{-9}M
SI 1.29x10^{-8}M
SII 1.39x10^{-7}M
SVdF 9.96x10^{-9}M
SBpA 1.21x10^{-8}M
DVG 8.85x10^{-8}M
Log[Analog] (M)
F
GRK6 siRNA - βarr$_{conf}$
EC$_{50}$
AngII 2.40x10^{-9}M
SI 7.19x10^{-8}M
SII 2.38x10^{-6}M
SVdF 2.47x10^{-8}M
SBpA 2.65x10^{-7}M
DVG 2.15x10^{-7}M
Log[Analog] (M)

Fig. 3. Role of GRK2 and GRK6 in βarrestin recruitment to AT1R and its change in conformation. Cells transfected with AT1R-YFP, βarr1-RlucII and control siRNA (A), GRK2 siRNA (C) and GRK6 siRNA (E) were stimulated with increasing concentrations of AngII and analogs for 25 min. Data are the mean ± S.E.M. of 3 independent experiments. Dose-dependent agonist-promoted decrease of βarrestin intramolecular BRET. Cells were transfected with HA-AT1R, GFP^{10}-βarr1-RlucII and control siRNA (B), GRK2 siRNA (D) and GRK6 siRNA (F) and treated as in A, C and E. The "x" represents the baseline signal from vehicle treated cells. Data are the mean ± S.E.M. of 3 independent experiments done in triplicate.

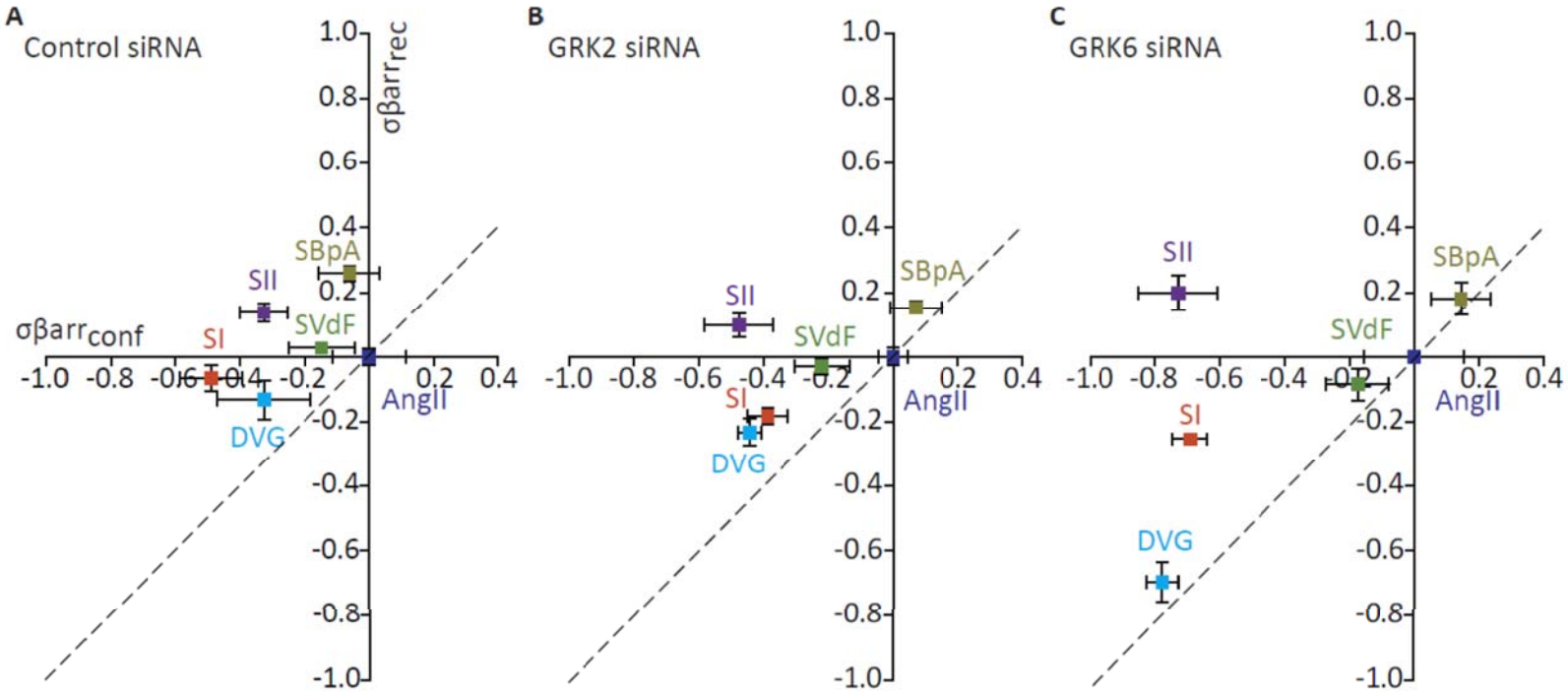
A
Control siRNA
σβarrrec
σβarrconf
SBpA
SII
SVdF
SI
AngII
DVG
B
GRK2 siRNA
SII
SBpA
SVdF
SI
AngII
DVG
C
GRK6 siRNA
SII
SBpA
SVdF
SI
AngII
DVG

Fig. 4. Sigma plots examining the effects of GRK2 and GRK6 on the signaling efficiencies of βarrestin.

A, Comparison of effective signaling (σ) in βarrestin conformation change and βarrestin recruitment for the analogs compared with the reference agonist, AngII, with control siRNA (A), GRK2 siRNA (B) and GRK6 siRNA (C). The broken line is the theoretical line of balanced signaling.

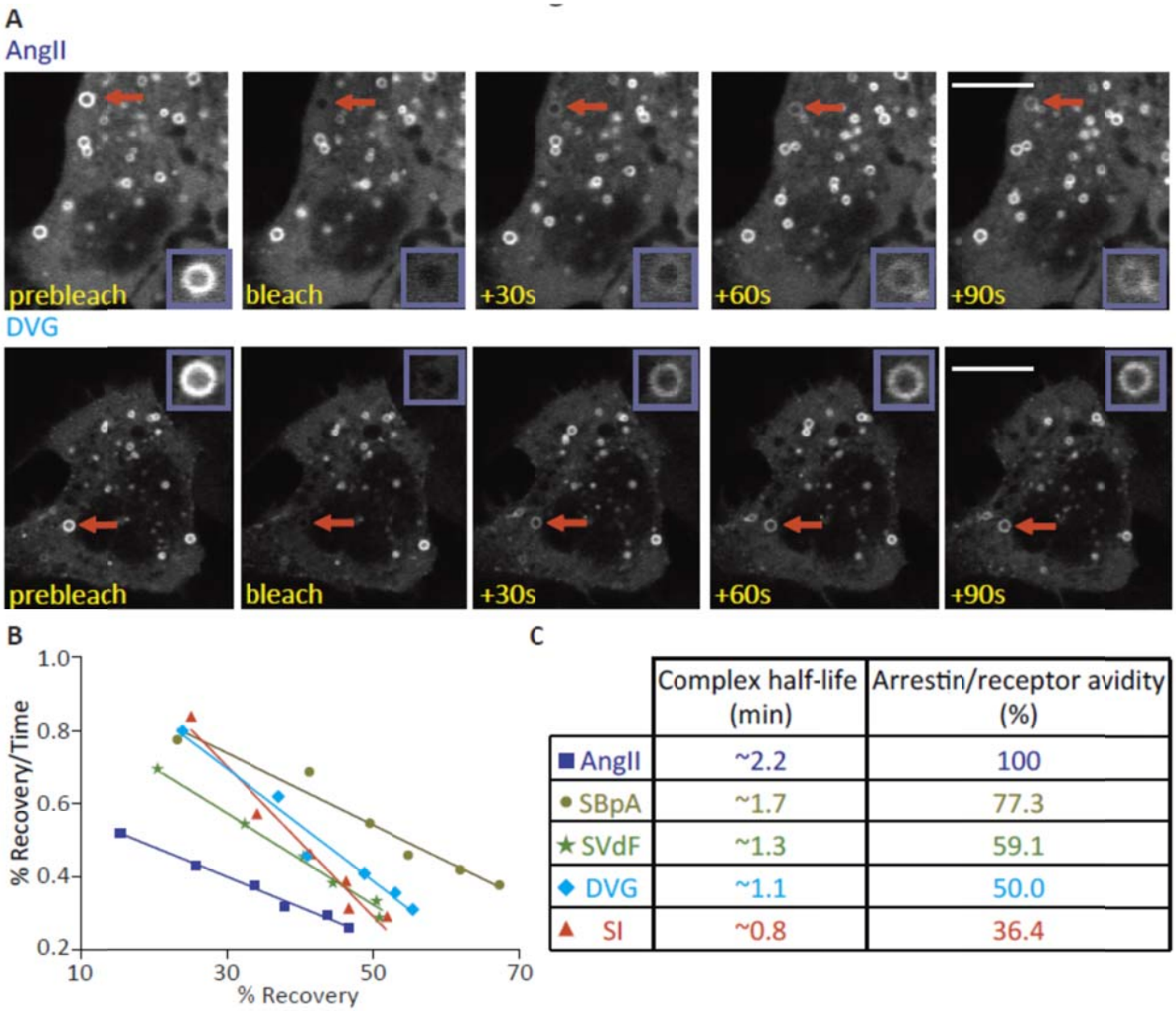

Fig. 5. Avidity of βarrestin for AT1R varies dependent on the ligand as revealed by FRAP.

A, Representative images of FRAP recovery experiment for AngII and DVG. HA-AT1R cells were transfected with βarr2-YFP. Cells were treated with 1μM AngII or 10μM of analog. After 15 minutes, endosomes were selected, bleached, and their fluorescence recovery rate was monitored as described in the Materials and Methods. Scale bar represents 10μm. B, Linear regression analysis of the recovery data representing an average of at least 7 endosomes per ligand. C, Recovery half-life was established based on the linear regression analysis. The slope of AngII was set to 100%.

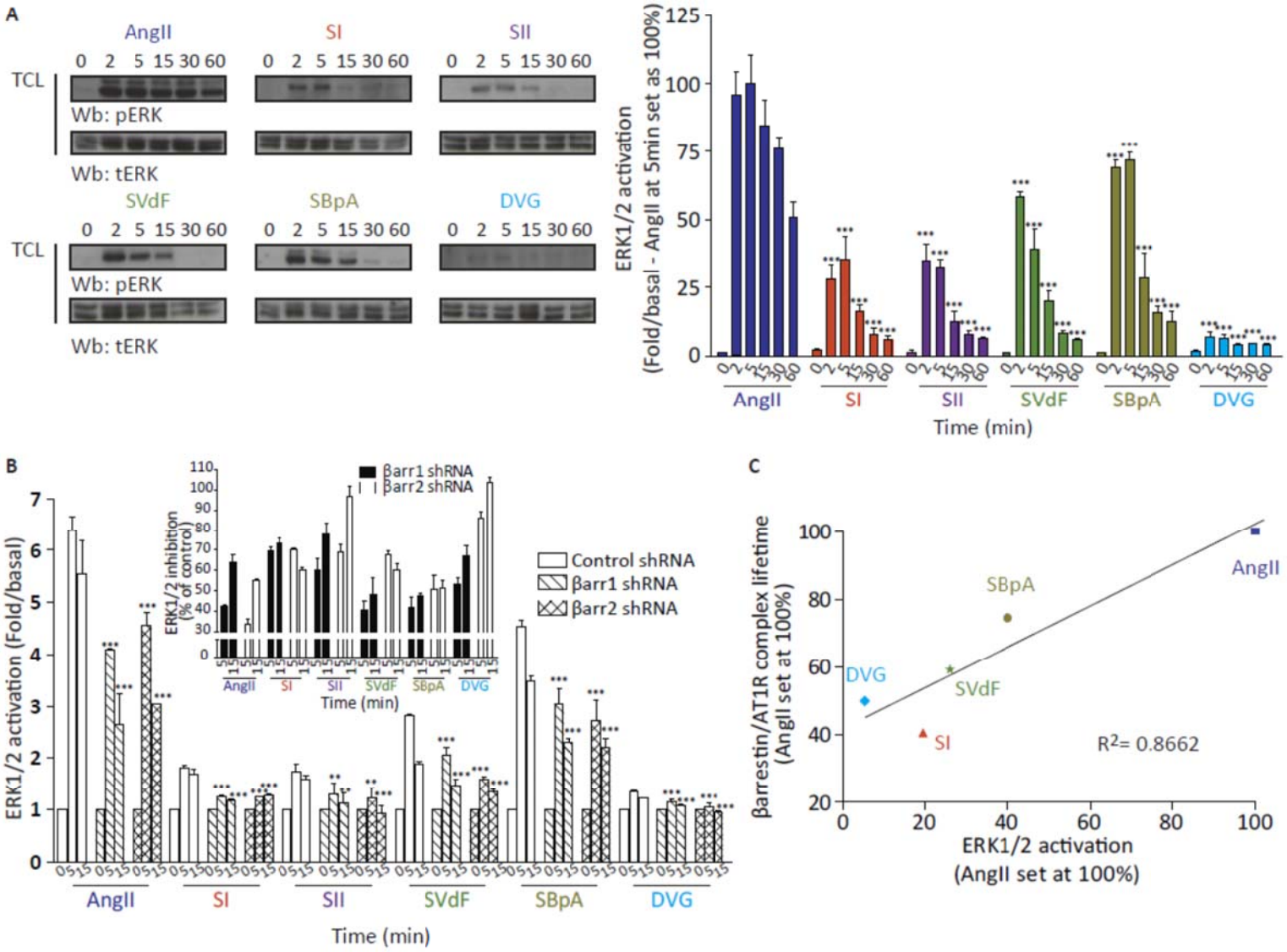
A
AngII
SI
SII
SVdF
SBpA
DVG
0 2 5 15 30 60
TCL
Wb: pERK
Wb: tERK
ERK1/2 activation
(Fold/basal - AngII at 5min set as 100%)
Time (min)
B
βarr1 shRNA
βarr2 shRNA
ERK1/2 inhibition
(% of control)
Control shRNA
βarr1 shRNA
βarr2 shRNA
ERK1/2 activation (Fold/basal)
Time (min)
C
βarrestin/AT1R complex lifetime
(AngII set at 100%)
ERK1/2 activation
(AngII set at 100%)
R2= 0.8662

Fig. 6. Regulation of ERK1/2 activation by the AngII analogs.

A, Effects of AngII analogs on MAPK activation. HA-AT1R cells were treated with 1μM AngII or 10μM analog for the times indicated. Lysates were immunoblotted for phospho-ERK and total ERK. pERK levels were normalized to total ERK and pERK at 5min treatment of AngII was set as the maximum. Shown is a representative blot. Quantification of results is the mean ± S.E.M and is representative of 5 independent experiments. Two-way ANOVA followed by a Bonferroni post-hoc test (with AngII as reference) was performed. B, Role of βarrestin in MAPK activation. As in A, except cells were transfected with shRNA against βarrestin1 or βarrestin2 and Perkin Elmer's AlphaScreen technology was used to measure ERK1/2 activation. Quantification is the mean ± S.E.M and is representative of 3 independent experiments, done in duplicate. Two-way ANOVA followed by a post-hoc Bonferroni's test. **, $p<0.01$, ***, $p<0.001$. Inset represents percent inhibition of ERK1/2 signal relative to shRNA control transfected cells. C, Correlation plot demonstrating the linear relationship between ligand-mediated ERK1/2 activation at 15 min and the avidity of the βarrestin and receptor complex.

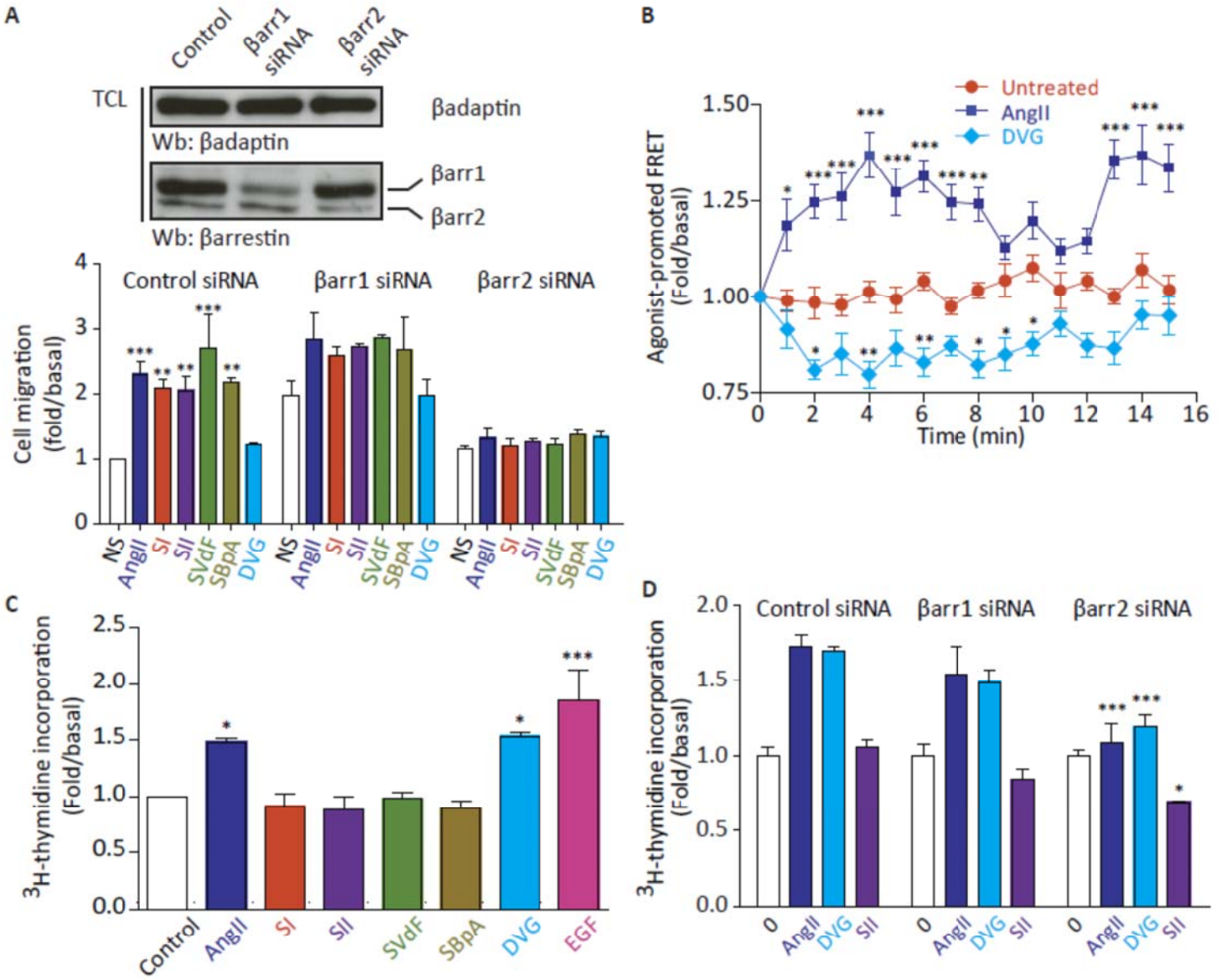
A
Control
βarr1 siRNA
βarr2 siRNA
TCL
βadaptin
Wb: βadaptin
βarr1
βarr2
Wb: βarrestin
Control siRNA
βarr1 siRNA
βarr2 siRNA
Cell migration (fold/basal)
NS
AngII
SI
SII
SVdF
SBpA
DVG
B
Untreated
AngII
DVG
Agonist-promoted FRET (Fold/basal)
Time (min)
C
3H-thymidine incorporation (Fold/basal)
Control
AngII
SI
SII
SVdF
SBpA
DVG
EGF
D
Control siRNA
βarr1 siRNA
βarr2 siRNA
3H-thymidine incorporation (Fold/basal)
0
AngII
DVG
SII

Fig. 7. AngII analog signaling in VSMCs reveals sub-receptor level βarrestin-dependent signaling bias.

A, VSMC migration assay in presence of siRNA against βarrestin. Cells were transfected with control, βarrestin1 or βarrestin2 siRNAs. Knockdown was verified 72h post transfection with β-adaptin as a control. TCL, total cell lysate and Wb, western blot. VSMCs were seeded into Boyden chambers and cells migrating to the lower chamber were counted. Results are representative of 4 independent experiments, presented as mean ± S.E.M.. One-way ANOVA followed by a Dunnett's post-hoc test (untreated for each condition as control) was performed. B, βarrestin conformational change by FRET in VSMCs. VSMCs transfected with AT1R-HA and CFP-βarr2-Venus were treated with buffer, 100nM AngII or 10μM DVG. Readings were taken every minute for 16 minutes. Signal from untransfected cells was subtracted and fold was determined by division of the pre-stimulated signal. Quantification of 6 independent experiments done in triplicate. Two-way repeated measures ANOVA followed by a Bonferroni post-hoc test was performed. C, Cell growth by ^{3}H-thymidine incorporation in VSMCs. ^{3}H-thymidine was added to VSMCs concurrent with stimulation (100nM AngII/10μM analog) for 24h. Thymidine incorporation was then assessed. Quantification of 6 independent experiments. One-way ANOVA followed by a Dunnett's post-hoc test (untreated as control) was performed. D, Role of βarrestin in cell growth by ^{3}H-thymidine incorporation in VSMCs. As in C, except cells were transfected with siRNA. Quantification of 3 independent experiments. Two-way ANOVA followed by a Bonferroni post-hoc test was performed (control siRNA compared to 3 experimental conditions). *, $p<0.05$, **, $p<0.01$ ***, $p<0.001$.

Table 1. Sigma (σ) values and bias factors (β) comparing IP1 vs. βarrestin recruitment to AT1R

The column "bias" denotes whether a statistically significant difference between the two pathways is seen in reference to the control compound, AngII.

IP1 vs. βarr_{rec}								
	σ_{IP1}	S.E.M.$_{\sigma IP1}$	$\sigma_{\beta arr\text{-}rec}$	S.E.M.$_{\sigma\beta arr\text{-}rec}$	β	S.E.M.$_{\beta}$	Bias	p-value
AngII	0.000	0.068	0.000	0.123	0.000	0.099	-	-
SI	n.d.	n.d.	0.230	0.076	n.d.	n.d	Bias	-
SII	n.d.	n.d.	0.644	0.061	n.d.	n.d	Bias	-
SVdF	-0.406	0.109	0.228	0.073	-0.449	0.093	Bias	<0.01
SBpA	-0.057	0.079	0.597	0.069	-0.462	0.074	Bias	<0.01
DVG	n.d.	n.d.	-0.120	0.114	n.d.	n.d.	Bias	-

Table 2. Sigma (σ) values and bias factors (β) comparing βarrestin conformation change vs. recruitment to AT1R

The column "bias" denotes whether a statistically significant difference between the two pathways is seen in reference to the control compound, AngII.

βarr_{conf} vs. βarr_{rec} - Control siRNA								
	$\sigma_{\beta arr\text{-}conf}$	S.E.M.$_{\beta arr\text{-}conf}$	$\sigma_{\beta arr\text{-}rec}$	S.E.M.$_{\sigma\beta arr\text{-}rec}$	β	S.E.M.$_{\beta}$	Bias	p-value
AngII	0.000	0.111	0.000	0.026	0.000	0.081	-	-
SI	-0.489	0.096	-0.065	0.040	-0.300	0.074	Bias	<0.01
SII	-0.327	0.075	0.137	0.028	-0.328	0.056	Bias	<0.01
SVdF	-0.146	0.104	0.027	0.013	-0.122	0.074	-	n.s.
SBpA	-0.059	0.094	0.259	0.024	-0.225	0.068	Bias	<0.05
DVG	-0.325	0.144	-0.132	0.060	-0.136	0.110	-	n.s.

βarr_{conf} vs. βarr_{rec} - GRK2 siRNA								
	$\sigma_{\beta arr\text{-}conf}$	S.E.M.$_{\beta arr\text{-}conf}$	$\sigma_{\beta arr\text{-}rec}$	S.E.M.$_{\sigma\beta arr\text{-}rec}$	β	S.E.M.$_{\beta}$	Bias	p-value
AngII	0.000	0.045	0.000	0.027	0.000	0.037	-	-
SI	-0.387	0.063	-0.181	0.025	-0.146	0.048	Bias	<0.05
SII	-0.477	0.107	0.097	0.037	-0.406	0.080	Bias	<0.001
SVdF	-0.218	0.085	-0.029	0.024	-0.134	0.062	Bias	<0.05
SBpA	0.070	0.080	0.153	0.020	-0.059	0.058	-	-
DVG	-0.444	0.037	-0.232	0.042	-0.150	0.039	Bias	<0.05

βarr_{conf} vs. βarr_{rec} - GRK6 siRNA								
	$\sigma_{\beta arr\text{-}conf}$	S.E.M.$_{\beta arr\text{-}conf}$	$\sigma_{\beta arr\text{-}rec}$	S.E.M.$_{\sigma\beta arr\text{-}rec}$	β	S.E.M.$_{\beta}$	Bias	p-value
AngII	0.000	0.156	0.000	0.010	0.000	0.110	-	-
SI	-0.693	0.052	-0.252	0.012	-0.312	0.038	Bias	<0.01
SII	-0.730	0.121	0.199	0.055	-0.657	0.094	Bias	<0.001
SVdF	-0.175	0.096	-0.085	0.050	-0.063	0.077	-	n.s.
SBpA	0.146	0.092	0.182	0.051	-0.025	0.075	-	n.s.
DVG	-0.777	0.049	-0.696	0.063	-0.057	0.056	-	n.s.

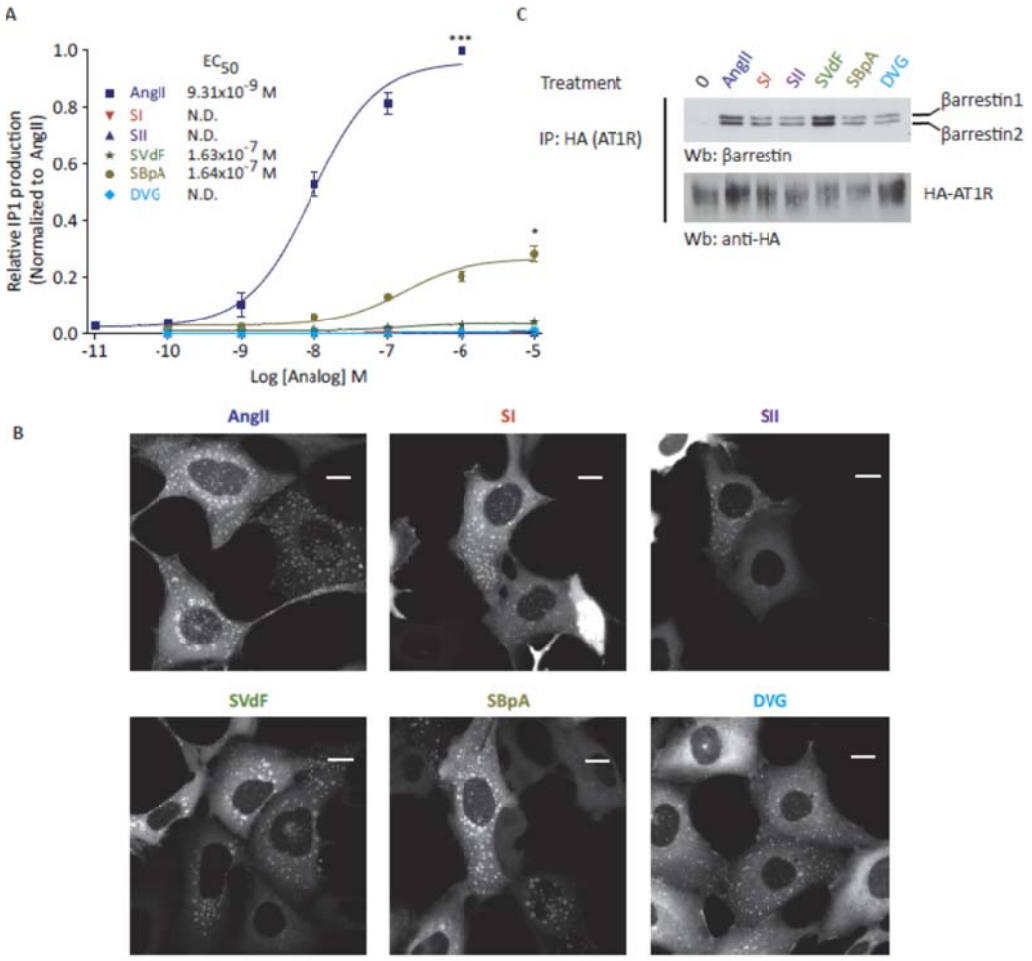

Figure S1. IP1 production of the analogs and recruitment of βarrestin by confocal microscopy and immunoprecipitation.

A, HEK293 cells stably expressing HA-AT1R were used to measure IP1 production downstream of the AT1R using CisBio's IP-one HTRF assay. Cells were treated with increasing concentrations of AngII or the analogs. IP1 production by AngII was set to be the maximum. Data is representative of 3 independent experiments performed in duplicate. Statitistical analysis was performed on maximal ligand concentration using a one-way ANOVA followed by a Dunnett's post-hoc test with untreated as control. *, $p<0.05$, ***, $p<0.001$. B, HA-AT1R cells transfected βarr2-YFP were stimulated with AngII (1µM) or analogs (10µM) for 15min. Images are representative of 3 independent experiments. Scale bars are indicative of 10µm. C, HEK293 cells stably expressing HA-AT1R were stimulated for 15min with either 1µM AngII or 10µM analog. Receptor immunoprecipitates were immunoblotted for βarrestin and AT1R. Representative blot is shown, n=3.

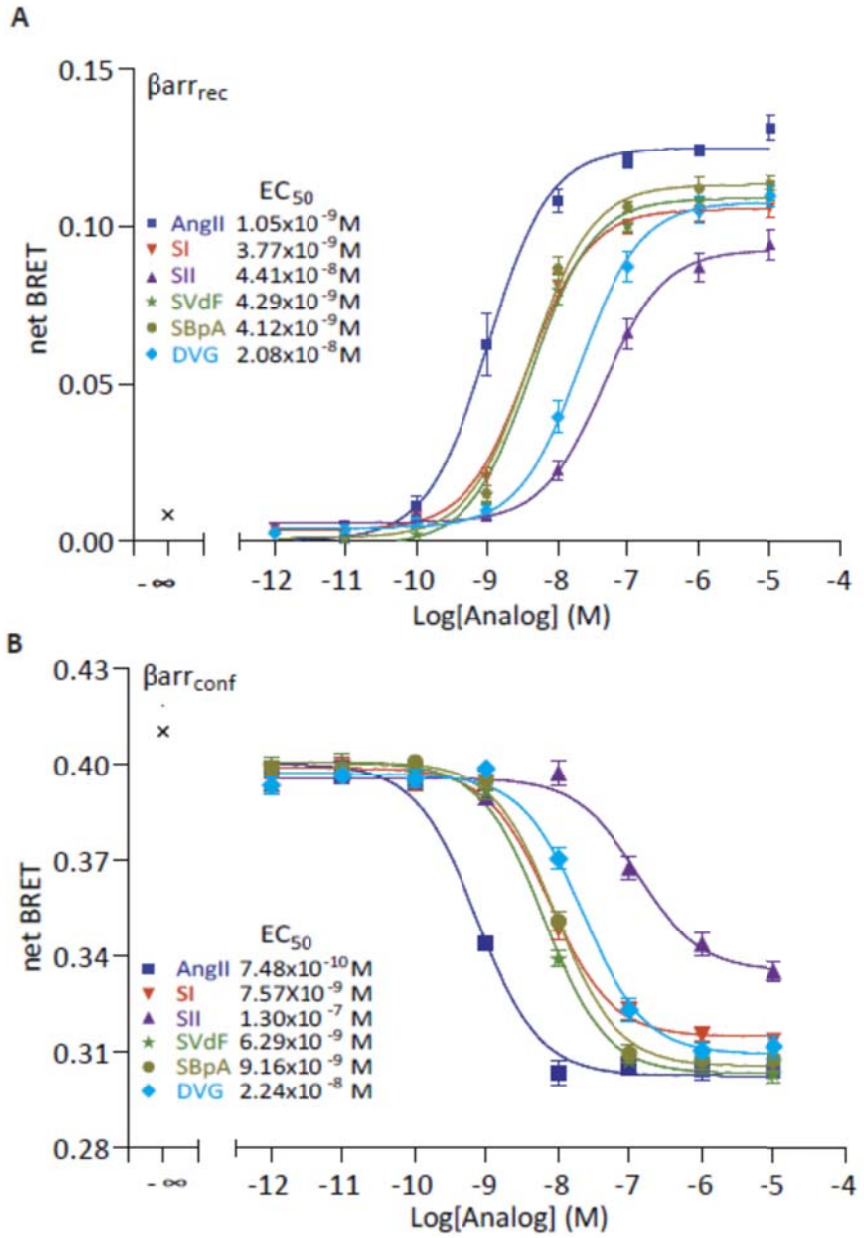

Fig. S2. Beta-arrestin recruitment to AT1R and its change in conformation by AngII analogs.

A, Dose-dependent agonist-promoted increase of βarrestin to AT1R BRET. A, Cells transfected with AT1R-YFP and βarr1-RlucII were stimulated with increasing concentrations of AngII and analogs for 25 min. The "x" represents the baseline signal from vehicle treated cells. Data are the mean ± S.E.M. of 3 independent experiments. Curves were fitted using nonlinear regression. B, Dose-dependent agonistpromoted decrease of βarrestin intramolecular BRET. As in A, but cells were transfected with HA-AT1R and GFP10-βarr1-RlucII.

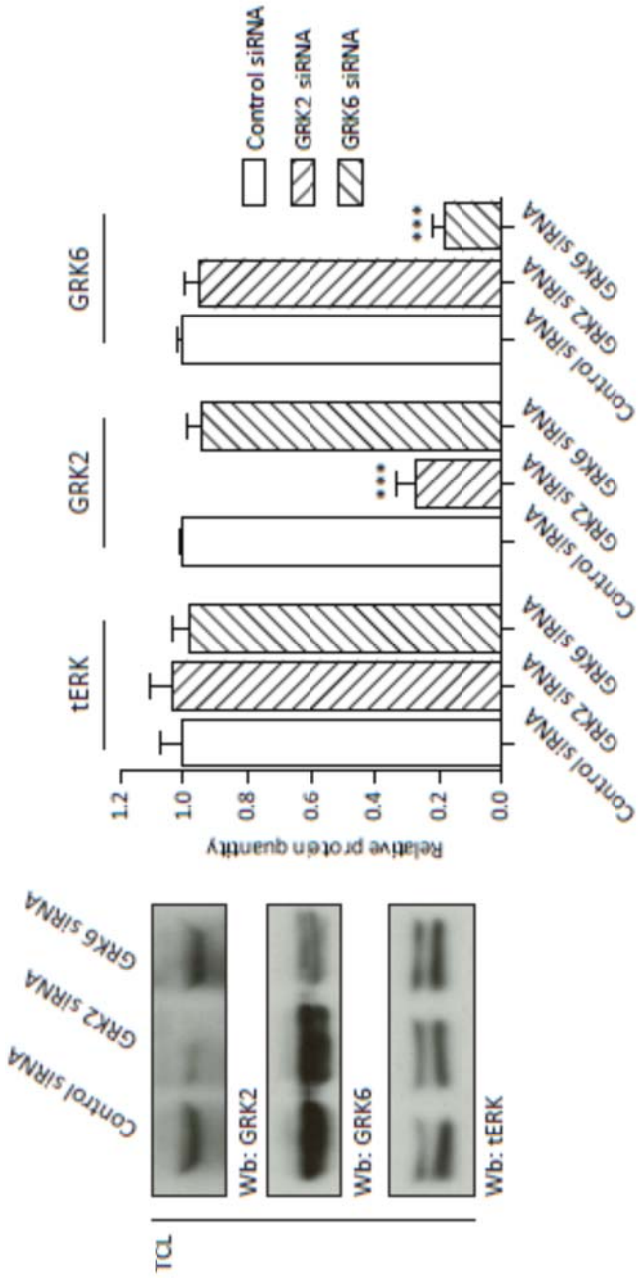

Figure S3. siRNA-mediated knockdown of GRK2 and GRK6.

Representative blot of GRK2 and GRK6 knockdown. Quantification of 3 independent experiments from cells used in BRET data presented in Fig. 3. tERK used as loading control. One-way ANOVA followed by a Dunnett's post-hoc test was performed. ***, $p<0.001$

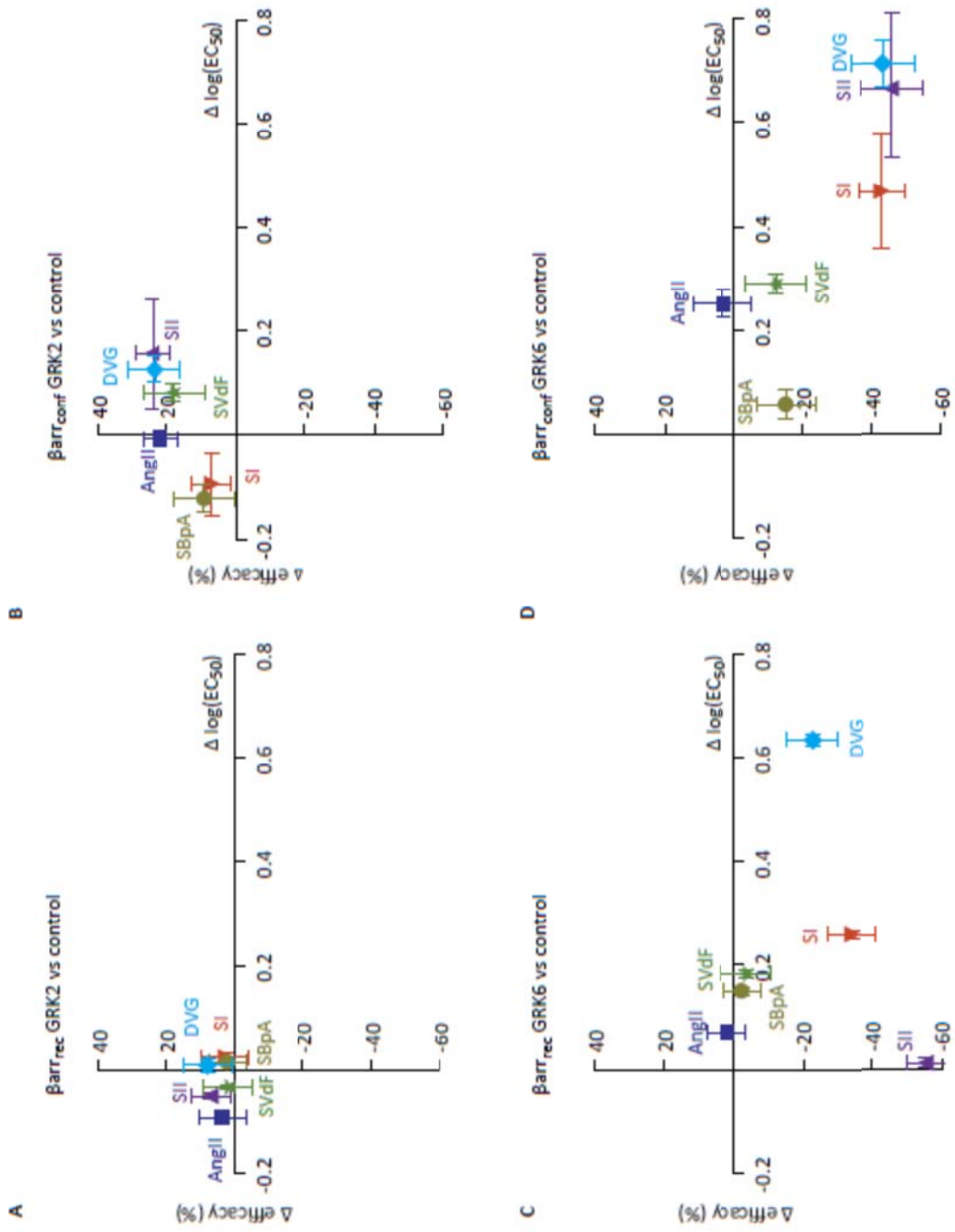

Figure S4. Role of GRK2 and GRK6 in the efficacy and potency on ligand-induced βarrestin recruitment and conformational change.

The log(EC50) and the efficacy of the analogs to induce βarrestin recruitment (A) or conformational change (B) were subtracted between the GRK2 knockdown and control conditions. The data are presented as the change in potency and efficacy. The same analysis was performed in C and D respectively, in reference to GRK6 depletion vs. control cells.

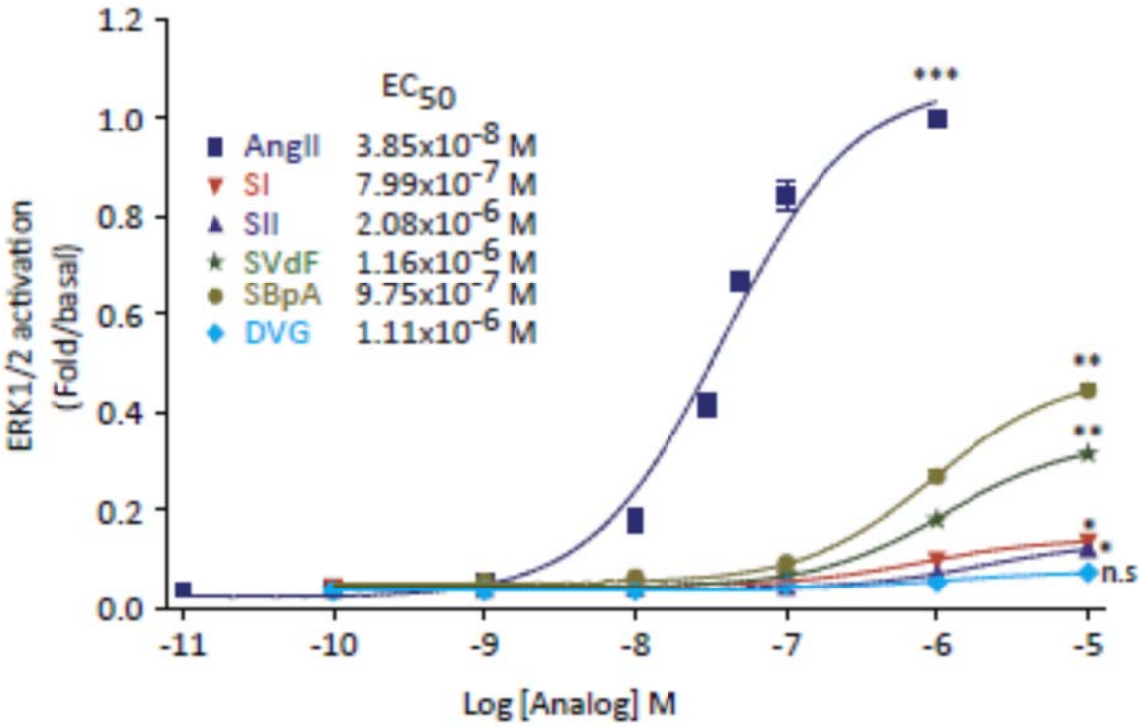

Figure S5. Dose-response relationship of ERK1/2 activation by AngII and analogs.

Perkin Elmer's AlphaScreen technology was used to assess the ERK1/2 activation mediated by increasing concentrations of AngII and the analogs. Data is representative of 3 independent experiments done in duplicate. Statitistical analysis was performed on maximal ligand concentration using a one-way ANOVA followed by a Dunnett's post-hoc test was performed with untreated as control. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, n.s., non-significant.

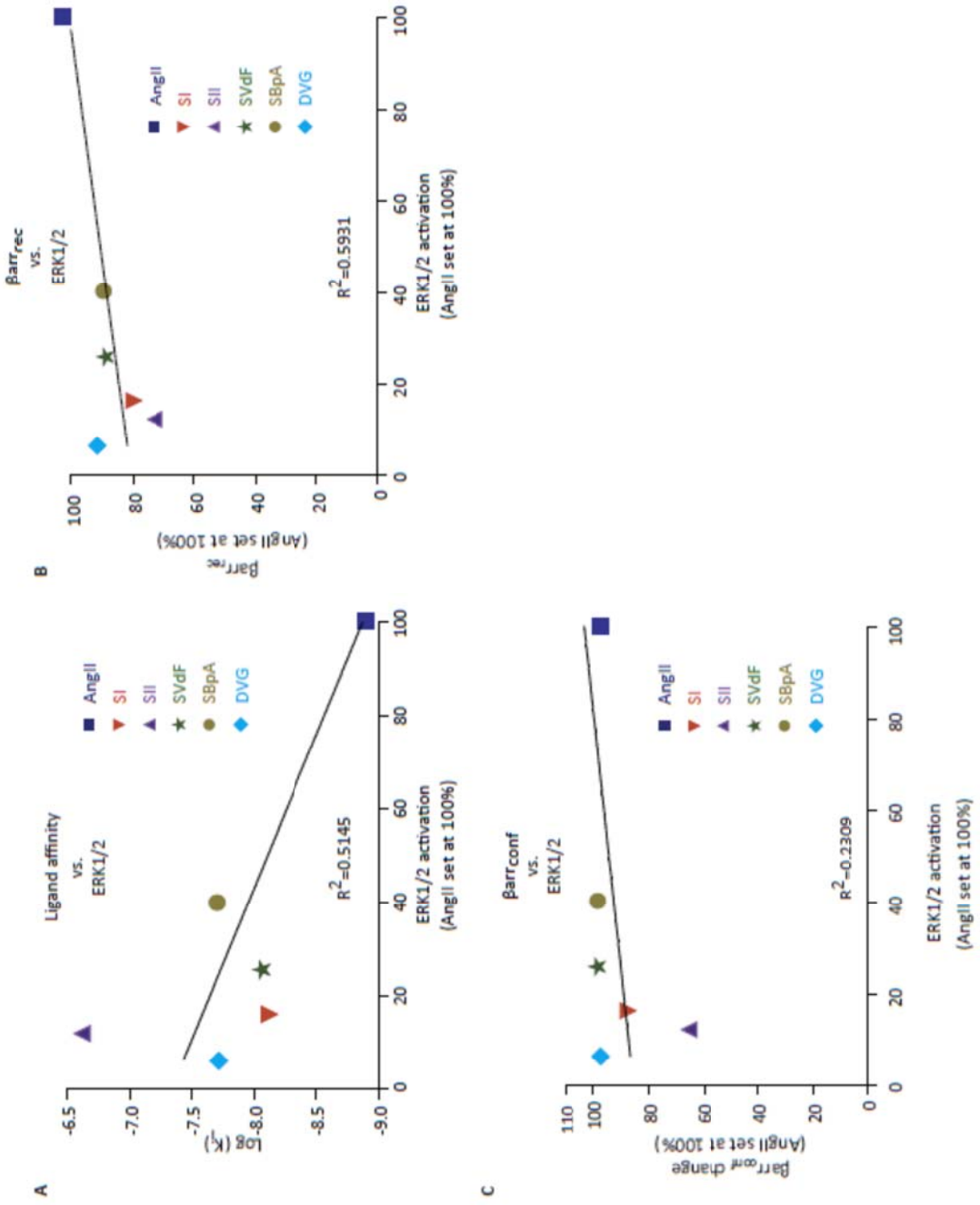

Figure S6. Correlation plots between ligand affinity, BRET values and ERK1/2 activation.

A, Values of ERK1/2 activation at 15min stimulation were correlated with the log of the Ki of binding. B, Values of ERK1/2 activation at 15min stimulation were correlated with the efficacy of βarrestin recruitment to AT1R by BRET. C, Values of ERK1/2 activation at 15min stimulation were correlated with the efficacy of βarrestin conformational change by BRET.

Ligand	Emax	LogKi	log(τ)	SEM log τ	R^2
Ang II	95.970	-8.938	0.919	0.068	0.981
SI	0.605	-8.155	n.d.	n.d.	0.451
SII	0.499	-6.672	n.d.	n.d.	0.353
SVdF	3.944	-8.101	0.513	0.109	0.852
SBpA	26.710	-7.749	0.863	0.079	0.918
DVG	1.078	-7.763	n.d.	n.d.	0.310

Table S1. IP1 dose-response curve data used for σ value and β factor calculation.

		βarr_{rec}			βarr_{conf}			
	Ligand	log(τ)	SEM log τ	R^2	Ligand	log(τ)	SEM log τ	R^2
Control siRNA	AngII	0.057	0.026	0.993	Ang II	-0.078	0.111	0.804
	SI	0.029	0.040	0.989	SI	-0.555	0.096	0.696
	SII	0.195	0.028	0.983	SII	-0.439	0.075	0.626
	SVdF	0.084	0.013	0.992	SVdF	-0.209	0.104	0.848
	SBpA	0.318	0.024	0.994	SBpA	-0.151	0.094	0.833
	DVG	-0.193	0.060	0.987	DVG	-0.391	0.144	0.840
	Ligand	log(τ)	SEM log τ	R^2	Ligand	log(τ)	SEM log τ	R^2
GRK2 siRNA	AngII	0.148	0.027	0.994	Ang II	-0.055	0.045	0.900
	SI	-0.076	0.025	0.984	SI	-0.492	0.063	0.778
	SII	0.238	0.037	0.953	SII	-0.486	0.107	0.601
	SVdF	0.119	0.024	0.988	SVdF	-0.287	0.085	0.860
	SBpA	0.301	0.020	0.990	SBpA	-0.058	0.080	0.875
	DVG	-0.122	0.042	0.991	DVG	-0.504	0.037	0.905
	Ligand	log(τ)	SEM log τ	R^2	Ligand	log(τ)	SEM log τ	R^2
GRK6 siRNA	AngII	-0.014	0.010	0.976	Ang II	-0.238	0.156	0.882
	SI	-0.267	0.012	0.952	SI	-1.046	0.052	0.546
	SII	0.175	0.055	0.826	SII	-0.876	0.121	0.160
	SVdF	-0.099	0.050	0.976	SVdF	-0.504	0.096	0.832
	SBpA	0.162	0.051	0.968	SBpA	-0.226	0.092	0.789
	DVG	-0.719	0.063	0.944	DVG	-1.046	0.049	0.629

Table S2. BRET dose-response data used for σ value and β factor calculation.

Chapter 5 –

Contribution to original research, discussion and conclusion

Contribution to original research

Chapter 2 – c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway

Here we demonstrated that in living cells $\beta_2$adaptin phosphorylation on tyrosine 737 occurred downstream of receptor activation and that the event was not confined to GPCRs as receptor tyrosine kinases, also elicited the event. This was accomplished through the generation and validation of a polyclonal antibody, raised specifically against this phosphosite. We further established the key factors involved in the phosphorylation event through knockdown studies and with biased ligands that selectively activate AT1R-mediated signalling. Two key proteins required for $\beta_2$adaptin phosphorylation were βarrestin and the kinase, c-Src. Overexpression of a dominant negative Src (K298R) or siRNA knockdown prevents $\beta_2$adaptin Y737 phosphorylation. We also localized spatially and temporally the phosphorylation event, as it occurred soon after receptor stimulation and was localized at the plasma membrane in clathrin-coated pits.

Chapter 3 – A functional role for Y737 of β_2-adaptin in the signalling and endocytosis of the AT1R revealed through an intrabody

While in chapter 2, we showed the importance of $\beta_2$adaptin Y737 phosphorylation in the dissociation of the AP-2/βarrestin complex, we were unable to reveal a consequence of the phosphorylation. In this study, we generated a single chain antibody generated against the phosphorylated Y737 that can be expressed intracellularly. Using this tool, we expose the functional significance of Y737 phosphorylation in live cells as playing a critical role in AT1R signalling and endocytosis. We also developed a non-phosphospecific antibody that recognized $\beta_2$adaptin in close proximity to tyrosine 737 (but does not include Y737 in its antigen), which allows us to compare effects of block AP-2

entirely versus blocking on the phosphorylated form. Our findings reveal that blocking unphosphorylated $\beta_2$adaptin does delay internalization of the receptor but unlike with the anti-pY737 antibody, the endocytic block eventually was overcome. Interestingly, the loss of signalling seen with expression of scFv-4B7A8, is not recapitulated at all by scFv-1E1F9. Importantly, we demonstrate that the signalling effect is not AT1R-limited as a similar block is seen with EGFR. To support our data, we performed similar experiments using a phosphorylation-deficient $\beta_2$adaptin (Y737F), and show that it displays similar effects to expression of the anti-phospho 737 intrabody. Finally, we have long speculated that Y737 in $\beta_2$adaptin is a binding site for SH2 domain containing proteins, due to the consensus motif proceeding Y737. GST pulldown experiments reveal that three SH2 domain containing proteins (p120Ras-GAP, Shc and p85β) bind to $\beta_2$adaptin in a phosphorylation-dependent manner. All three of these proteins play critical roles in receptor endocytosis and signalling.

Chapter 4 - Biased Signalling by angiotensin analogs leads to differential βarrestin-dependent cellular responses

In **Chapter 2**, we used a biased ligand to selectively activate the βarrestin-dependent pathway downstream of the AT1R. This led us to examine the signalling properties of four AngII analogs and compare their effects to AngII and the literature AT1R-biased ligand, SII. Our study revealed that we had uncovered three newly characterized AngII analogs that displayed a complete bias towards the βarrestin pathway, while the fourth analog appeared to a balanced, partial agonist. We proceeded to study the ligands' abilities at inducing conformation change of the endocytic adaptor, βarrestin, in correlation with the degree of recruitment. Here we were surprised to find that not only were the ligands able to induce differential conformations on βarrestin but that the degree of conformational change varied significantly between the ligands. We also

examine the role of GRK2 and GRK6 in this paradigm, and reveal differential roles for either kinase in recruitment and/or conformational change dependent on the ligand used. Furthermore, we established a correlation between the affinity of βarrestin for the receptor and the degree of receptor-mediated ERK activation, a link that has never been previously recognized. Finally, we tested the AngII analogs in a physiologically relevant model and find that despite all ligands being capable of βarrestin-dependent signalling, one ligand induced a different cellular outcome compared to the rest. Using a FRET based approach, we showed this ligand induces a different βarrestin conformation compared to the other ligands, which we proposed is what leads this ligand to have a unique output. To our knowledge, we are the first to demonstrate biased signalling at the sub-receptor level, where the endocytic adapter is targeting specific cellular outcomes based on its conformation.

Discussion and conclusion This thesis' aim was to uncover functional novel functional roles for endocytic adaptors in the signalling and endocytosis of GPCRs, specifically the angiotensin type I receptor. The work led to the discovery of a novel function for the clathrin adaptor, AP-2, implicating it as a signalling scaffold. Furthermore, we are the first to demonstrate biased signalling at a level below the receptor, where βarrestin signalling can determine independent functional outcomes based on its conformational change.

Chapter 2 and 3 explore the post-translational modification of the β-subunit of the clathrin adapter AP-2 and its impact on receptor signalling and trafficking. Previous work in our lab demonstrated that in response to AngII treatment, AT1R recruited βarrestin, Src and $\beta_2$adaptin/AP-2 and that they were critical components in proper receptor trafficking [1]. We went on to show that the putative phosphorylation at $\beta_2$adaptin tyrosine 737 regulates the interaction of the βarrestin/AP-2 complex, although no functional significance was attributed to why complex destabilization was relevant in receptor function [2]. The lack of functional significance was surprising as numerous studies had demonstrated that disrupting components of the clathrin-mediated endocytosis, whether it was βarrestin, AP-2 or dynamin resulted to a partial or total inhibition of endocytosis [3-5]. At the time, only one other tyrosine had been shown to be phosphorylated in $\beta_2$adaptin, tyrosine 6 following EGFR stimulation [6]. This group also found no relevant role for this phosphorylation event, potentially signifying $\beta_2$adaptin phosphorylation regulates processes that we have not explored yet. The study found in **chapter 2** focused on demonstrating that the $\beta_2$adaptin Y737 phosphorylation occurred in living cells, as all evidence at prior to this stage was obtained through mutagenesis studies and *in vitro* biochemical assays. Using a polyclonal rabbit antibody that we characterized and developed, we demonstrated specific recognition of phosphorylated $\beta_2$adaptin that was lost with Y737 substitution to phenylalanine. Using this tool, we revealed that AngII-mediated AT1R activation led to Y737 phosphorylation in multiple cells types.

Furthermore, we established the event as a general receptor mediated event, as GPCRs internalizing through the clathrin-dependent endocytic pathway induced the phosphorylation, whereas a GPCR known to internalize via a caveolin-dependent pathway did not. Surprisingly, the phosphorylation was also found by EGFR activation, which had been missed by the previous group [6], suggesting that this was a general receptor-mediated event associated with receptor endocytosis.

Given the limited tools at our disposal at this stage, we were unable to demonstrate any cell process that was regulated or modified by this phosphorylation event. To gain a better understanding of $\beta_2$adaptin Y737 phosphorylation's role, we decided to develop a method to express an antibody intracellularly to affect the phosphorylation event in real-time The path to the development of the intracellular antibody, which was to be a single chain antibody, was long and required the intermediate synthesis of the monoclonal antibodies. We were eventually successful in developing the scFvs, which allowed us to ascribe a functional importance to Y737 phosphorylation. Using this tool, we revealed this tyrosine's importance in both receptor-mediated signalling and endocytosis; a novel role for the clathrin adaptor AP-2 as a signalling scaffold and a complete block of receptor endocytosis. Our evidence supporting this claim centered on data demonstrating that the scFv that binds to phosphorylated Y737 or the substitution of this residue with a phenylalanine led to a 50% reduction in receptor mediated signalling. Also, we see a complete block of receptor endocytosis using the antibody against the phosphosite, whereas the total $\beta_2$adaptin intrabody has a much lesser effect. Furthermore, we demonstrate that $\beta_2$adaptin can bind to proteins containing SH2 domains, which are significantly involved in numerous signalling paradigms. This potential discovery is eerily reminiscent of the one for βarrestin, where for many years its ascribed function was solely to desensitize the receptor, inhibiting further receptor signalling and eventually lead to receptor endocytosis. However, later

studies began to recognize the ability for βarrestin to scaffold signalling proteins like the tyrosine kinase Src, revealing a signalling role for βarrestin [7-9].

There are alternates possibilities of how expression of the single chain antibody is modulating the receptor-mediated signalling. While we propose that $\beta_2$adaptin may be a signalling scaffold, we have no way of knowing the percentage of ERK1/2 activation it accounts for experimentally. Indeed, it is possible that the ERK activation that is lost by antibody expression or by expression of the phenylalanine-substituted $\beta_2$adaptin involves a decrease in other signalling pathways that are disrupted including G protein or βarrestin signalling. Studies attempting to determine the level of $\beta_2$adaptin-mediated ERK1/2 will have to be pursued. A secondary alternate explanation for the loss of ERK1/2 signalling could also be related to the inhibition of endocytosis, as it has been shown that for some receptors internalization is required for their resensitization. One study demonstrated that if β_2-adrenergic receptor recruitment of βarrestin was disrupted or if dynamin-K44A was expressed thus preventing receptor endocytosis, receptor signalling was negatively affected due to improper resensitization [4]. Therefore, it is possible that the loss of activation is due to the sustained desensitization of the AT1R or EGFR preventing proper signalling. Although our study examines the initial challenge of the receptor, therefore it points towards $\beta_2$adaptin as a signalling scaffold rather than a loss of receptor resensitization.

Our study implicates $\beta_2$adaptin as a potential target for the modulation of receptor signalling in instances where it may be aberrantly regulated. The single chain antibody specifically targeting $\beta_2$adaptin on Y737 has the potential to downregulate this uncontrolled signalling, and thus could be of potential therapeutic use in pathophysiological conditions where we would like to dampen the signalling. Overactivation of receptor signalling is a frequent characteristic of many different forms of cancer where overexpression or dysregulation of signalling molecules play a significant role in cancer cell progression. In fact, in

many forms of cancer, there is hyperactivation of tyrosine kinases. As we propose $\beta_2$adaptin as a signalling scaffold requiring phosphorylation on Y737, there is the possibility that AP-2 may be involved in a cancerous phenotype. Indeed, two large scale phosphoproteomic studies revealed that only Y737 of $\beta_2$adaptin of the over 30 tyrosine residues in its primary sequence displayed significantly increased levels of phosphorylation [10, 11]. Therefore, it is possible that its hyperphosphorylation interferes with normal receptor endocytosis in these cells. Furthermore, there is potential that AP-2 may directly contribute to the cancer phenotype through its potential role as a signalling scaffold. For these reasons, our scFv against phosphorylated Y737 of $\beta_2$adaptin may have therapeutic implications, as it can be used to localize cancer cells and perhaps even inhibit their growth.

Numerous studies have implicated the EGFR in multiple cancers including lung [12], breast [13, 14], colon [15], among others. Two monoclonal antibodies are actually available in clinic as chemotherapeutic agents that target EGFR/ErbB1 (Cetuximab) [16] and HER2/ErbB2 (Trastuzumab) [17] that act to bind the receptor's extracellular domain preventing their activation. While these represent more targeted approaches, theoretically inhibition of receptor mediated endocytosis using our single-chain antibody could have a general effect on receptor internalization and signalling. Therefore, targeting of the antibody to the pharmacologically relevant site of action would be critical prior to any potential use.

While there remains much to uncover about the importance of tyrosine 737 in $\beta_2$adaptin, it appears like a promising target to partially suppress receptor-mediated signalling. However, numerous studies still need to be done, first and foremost confirming the protein or proteins that are recruited to pY737 $\beta_2$adaptin in living cells. The three putative binding partners all have exciting potential due to their diverse and significant roles in signalling and endocytosis. In order to confirm binding of p120Ras-GAP, Shc or p85β, co-

immunoprecipitation experiments will have to be performed. Additionally, fusions protein of those above tagged to a fluorescent protein like mRFP can be used to verify colocalization with $\beta_2$adaptin (or AP-2) following receptor activation. Furthermore, BRET studies between $\beta_2$adaptin and the SH2 domain containing proteins would further substantiate any interaction we uncovered. Once the binding partner was confirmed, the study should go on to examine the functional effects of knocking down this protein, which in some respects should mimic the result of overexpression of our single chain antibody. Ideally, the study should eventually move into animal models, where targeted infection or expression of the antibodies in specific tissues could reveal potential therapeutic uses for the inhibition of Y737 $\beta_2$adaptin-mediated signalling.

Chapter 4 of this thesis shifted direction from the AP-2 complex and its regulation, and focused on the more prominent GPCR endocytic adaptor, βarrestin. While the importance of βarrestin was originally thought to lie with its functions as a desensitization protein and endocytic adapter, it has now become appreciated for its diverse role as a signalling mediator due to its scaffolding potential. βarrestin has been found to be a critical regulator of mitogen activated protein kinase signalling, as it can scaffold and directly lead to the activation of ERK and JNK [18, 19]. Further highlighting the diversity of βarrestin was the discovery that non-GPCRs like IGF1R [20] can recruit βarrestin, which altered the downstream signalling of the receptors. For IGF1R, βarrestin recruited served as an intermediary scaffolding protein to recruit the ubiquitin ligase, MDM2, resulting the downregulation of the receptor [21] and potential dysregulation of the cell cycle. Interestingly, it has also been demonstrated GPCR-mediated transactivation of receptor tyrosine kinases, can also rely on βarrestin. It is extremely clear in the case of the β_1-adrenergic receptor, which is a critical for heart muscle contraction, where the transactivation of EGFR through its activation confers cardioprotective effects on to the smooth muscle cells [22]. In fact, a drug currently in the clinic, which was originally thought to be solely an

antagonist of the β_1-adrenergic receptor, carvedilol, is now known to be more beneficial in cases of heart failure compared to other β-blockers for its ability to selectively active receptor-mediated βarrestin-dependent signalling (transactivation) without activating other signalling events [23].

The importance of βarrestin and its signalling functions really came to the forefront of GPCR signalling with the realization that activation of a GPCR was not an on and off switch, but that you could stimulate a GPCR and select the signalling pathway or pathways to be activated. This phenomenon is now referred to as ligand-directed or ligand biased signalling. The study presented in **Chapter 4** was designed to further explore and understand the individual signalling properties of the AT1R through the use of bias ligands. At the time of the studies inception, only one ligand had been well characterized for the AT1R besides AngII, which was the AngII analog, [Sar^1,Ile^4,Ile^8]-AngII or SII. Numerous studies had demonstrated its ability to solely engage the AT1R in βarrestin-dependent signalling without any activation of the $G\alpha_q$ [24-27]. However, it was clear that while SII was indeed a biased ligand, it was also a partial agonist of the receptor, and so at maximum SII-mediated βarrestin signalling was 50% that of AngII [28]. One aspect of our original study was to uncover a novel biased ligand of the AT1R that behaved more similar to AngII in the levels of βarrestin-mediated signalling. Unfortunately, during the course of our study a pharmaceutical company, Trevena, published an article describing a new AT1R biased ligand that had similar efficacy to AngII. They named their ligand, TRV120027, however it too is a AngII analog, [Sar1,D-Ala8]-AngII. While at this time, we had already discovered our own [Sar1,Val5,D-Phe8]-AngII, the manuscript was not ready for publication in other facets.

Despite the setback, we instead shifted the focus of our study towards understanding ligand bias, and attempting to determine what allowed for it to occur. Multiple studies had demonstrated that stimulation with different ligands could induce differential conformations upon βarrestin [29, 30]. Therefore, we

wondered whether there existed distinct conformations for βarrestin upon its recruitment to the receptor using our analogs, and what roles GRK2 and GRK6 would play in the conformation and recruitment of βarrestin. While we see differential conformational change between the ligands, much of this could be explained through altered levels of recruitment. However, we revealed differential roles for GRK2 and GRK6 in both βarrestin recruitment and conformational change. Indeed, for some ligands, GRK6 was primarily involved in recruitment of βarrestin, whereas it was involved for conformational change for another. These results highlight a possible mechanism for biased signalling. Therefore, future work here will need to explore the method specific ligands induce differential conformations. There exist numerous possibilities here including differential receptor conformation, post-translation modification of the receptor and/or βarrestin itself, or even recruitment of different accessory proteins in concert with βarrestin. A recent study supports the potential of post-translational modification being critical in βarrestin conformation, as they demonstrated that alteration of the phosphorylation of the GPCR by GRKs could result in different βarrestin shapes [31, 32]. It is likely that the conformation of βarrestin will depend on a variety of factors with each playing a part in the overall shape of βarrestin.

While future work will have to address the mechanism of this phenomenon in our system, our results point to the latter case where it seems likely that the altered βarrestin conformation is the result of or results in the recruitment of alternative binding partners. This conclusion is drawn from our data in vascular smooth muscle cells where although all the ligands are not activating G protein signalling (Figure 1), yet maintain βarrestin signalling the majority of ligands mediate βarrestin-dependent cell migration, while only DVG mediates βarrestin-dependent cell growth. Transactivation of the EGFR is integral for the VSMC growth (Figure 2) but we did not establish the pathway involved in cell migration. In these cells, we were able to demonstrate that DVG

caused an alternate conformation of βarrestin compared with AngII, suggesting that its unique signalling potential was indeed the result of conformational signalling. A recent study demonstrated that βarrestin1 could recruit ARHGAP21, a GAP responsible for the inhibition of RhoA, a critical factor in the cell cytoskeleton [33]. While βarrestin does not interact with this GAP, it does highlight the potential for the recruitment of proteins that regulate these processes. Future work here will need to focus on determining the mechanism for each cellular outcome, *i.e.* cell growth or migration, by demonstrating differential recruitment of accessory proteins to βarrestin and their involvement in the role in both processes.

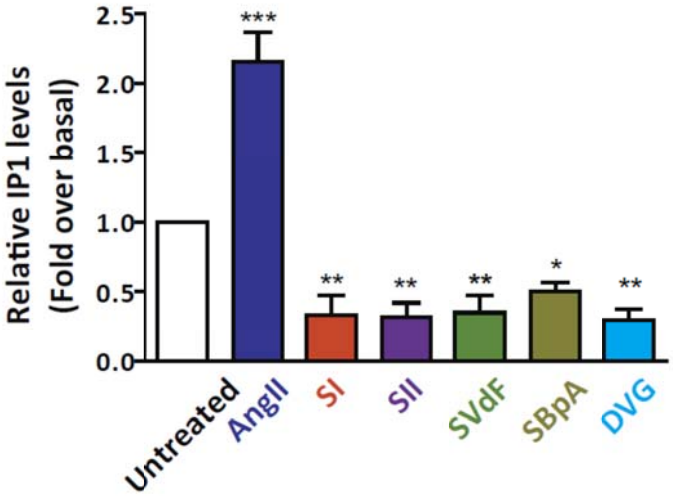

Figure 1. IP1 levels induced by the AngII analogs in VSMCs signifying G protein coupling.

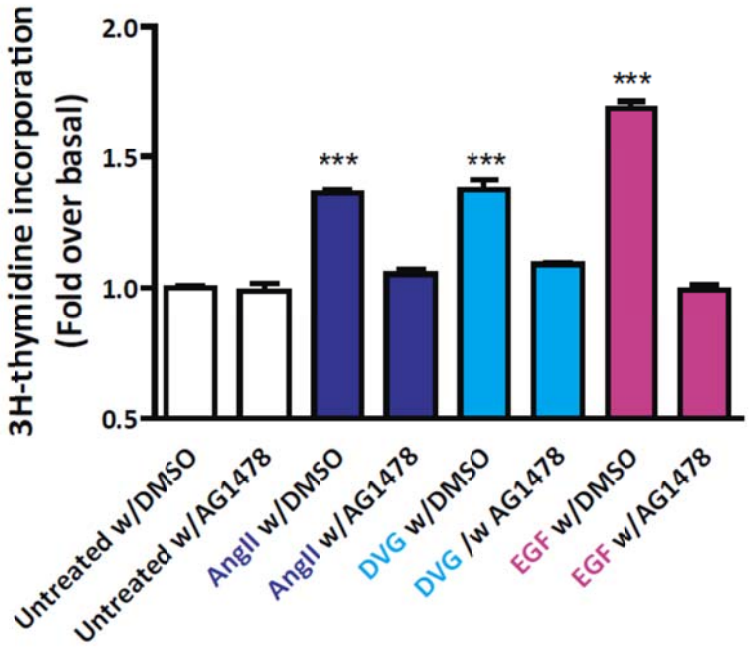

Figure 2. AT1R-mediated cell growth in VSMCs is dependent on EGFR-transactivation.

The potential benefits of biased signalling in the clinic are really just at its infancy with few known drugs in clinic functioning as selective ligands to our knowledge. As mentioned above, the β-blocker, carvedilol, is the only currently approved therapeutic that has been demonstrated to potentially have improved therapeutic efficacy as a result of its biased signalling. However, much promise is emerging in the field and a significant amount of resources is being devoted by multiple companies to explore its therapeutic potential. The AT1R is at the forefront of this advancement with the most recent AngII analog, TRV120027, actually reaching phase 2 clinical trials for acute heart failure. Preclinical studies demonstrated that TRV120027 resulted in improved cardiac output and renal protection compared to the conventional AT1R treatment, angiotensin receptors blockers (ARBs) [34, 35]. Therefore, much promise exists for biased ligands in therapeutics and more recent work is focusing on the μ-opioid receptor. While the future for drug design is exciting, it is still unclear how to rationally design biased ligands. Furthermore, as demonstrated in our study the ligand bias can

not only be at a pathway directly beneath the receptor, *i.e.* G protein or βarrestin but can be at a sub-level within βarrestin's own signalling. Therefore, the development of these drugs may be more complicated than envisioned, as it is possible that two drugs selectively engaging βarrestin-dependent signalling will not have the same functional outcome.

While there exists much work to explore the mechanistic details of both the single chain antibody and βarrestin conformational dependent signalling, I feel the contributions and advancements that I made to the projects have been significant. Both projects are now at an exciting location, where the fundamental basis for two uncharacterized processes can be explored. The discovery that $\beta_2$adaptin and AP-2 can function as a signalling scaffold complicates receptor-mediated signalling by adding an additional component that will almost always need to be considered. Furthermore, the revelation that βarrestin signalling can be altered based on its conformation, complexifies the landscape of general receptor and cellular outcomes.

Future work

While some future work was outlined in the above discussion, this section will briefly summarize the direction I hope the projects will take. The results presented here pertaining to the scFv against phosphorylated Y737 $\beta_2$adaptin open the door to exciting potential for AP-2. Its current role is believed to be well-defined as an endocytic adaptor for clathrin; however, the potential for it to act as a signalling scaffold could change how we view receptor signalling and internalization in general. Experiments first need to focus on establishing what proteins are actually signalling adaptors for AP-2 and determining the mechanism of AP-2-mediated signalling. Also, these signalling proteins may play critical roles in receptor endocytosis, as we see a complete block of internalization in the presence of 4B7A8-scFv. Understanding how endocytosis is blocked by the antibody is critical. If successful, it would be interesting to see what pathways are activated as a direct result of $\beta_2$adaptin scaffolding, and to

establish what physiological input this signalling had. Finally, as mentioned above Y737 of $\beta_2$adaptin has been shown to be hyperphosphorylated in two large scale phosphoproteomic studies, therefore, the effects of the scFv expression can be examined in cancer cell line models. While it is unclear what the results of expression would be, one could speculate a few potential scenarios. The antibody may bind onto phosphorylated $\beta_2$adaptin inhibiting receptor endocytosis in these cells, and potentially leading to the desensitization and eventual downregulation of the receptor. Alternatively, it may actually inhibit AP-2 mediated signalling, which potentially are involved in the cancer phenotype itself, and thus antibody treatment may represent a potential therapy. At minimum, the antibody may be useful as a biomarker, recognizing only cancerous cells due to their increased phosphorylated $\beta_2$adaptin levels.

The biased signalling section described here is much more complete; however, it too can be further developed. While we demonstrated that differential signalling may be the result of altered βarrestin conformation, the evidence is only correlative. In order to definitively demonstrate conformational biased signalling, the alternative binding partners need to be discovered. Experimental results would reveal that when the receptor is stimulated with one AngII analog compared to another that complexed proteins with βarrestin would vary. This could be accomplished with proteomic analysis of interacting proteins and highlighting those that bind in the presence of the different ligands. While this is quite doable, other future work could prove significantly more challenging. We still do not know how the ligands lead to receptor biased signalling. While it is readily assumed that it is relies on the conformation of the receptor, it is unclear how that leads to selective signalling. While structural studies of the receptor in complex with the different ligands will give context about the receptor, a daunting task on its own, it would still not likely reveal how the receptor selectively activates downstream pathways. Co-crystallization of the receptor with G proteins or βarrestin in the absence or presence of ligand is

essential for revealing the determinants for ligand biased signalling. Given that structural information it may allow the rational design of biased therapeutics.

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