

Characterization of β -arrestins Trafficking and Signaling Functions on G Protein-Coupled Receptors

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“The two most important days in your life are the day you are born
and the day you find out why”

-Mark Twain

Abstract

The heptahelical G protein-coupled receptors (GPCRs) are the largest, most versatile superfamily of cell surface receptors and constitute the most common target for many therapeutic drugs. They participate in various physiological processes via signaling transduction, mainly induced by heterotrimeric G proteins. Following activation, these 7-transmembrane receptors recruit β -arrestin for endocytosis. Beta-arrestins will directly associate GPCRs to several components of the endocytic machinery, such as clathrin and adaptor protein 2 (AP-2), allowing the receptor to internalize. Over the years, β -arrestins were shown to also act as signaling adaptors upon binding to agonist-occupied GPCRs; however, the mechanism regulating receptor/ β -arrestin complexes in endosomes was not yet addressed. Based on a previous study where we showed that β -arrestin serves as a scaffolding protein for several signaling effectors (e.g. MAPK) in the endosomes, we hypothesized that endosomal MAPK activity would be necessary to maintain the GPCR/ β -arrestin-2 complex, thus regulating receptor trafficking.

Our first study revealed a putative phosphorylating MAPK motif (Thr178) in β -arrestin-2 suggesting that endosomal MAPK activity is involved in such process. Using biochemical assays and FRAP (Fluorescence Recovery After Photobleaching) approach to assess the life-time of bradykinin B2 receptor (B2R)/ β -arrestin-2 endosomal complexes, we showed that the MAPK putative site in β -arrestin-2 is involved in regulating the association between the arrestin and its receptor. The role of β -arrestin-2 'hinge' domain was tested for such effect, and using arrestin mutants demonstrated distinct behaviours between β -arrestin-2 species on GPCRs trafficking and agonist-

mediated receptor signaling. This study highlights a strong correlation between MAPK signaling and the regulation of endosomal GPCR/ β -arrestin-2 interactions.

In addition to the ‘hinge’ domain, structural studies showed that the polar core, as well as the arrestins loops, were also crucial for β -arrestin activation. Based on the crystal structure of β -arrestin-1, a virtual screen was then conducted in order to identify a selective pharmacological inhibitor of β -arrestin-2. Results showed a unique compound, namely UM0012685 which firstly, blocked V2-vasopressin receptor (V2R) endocytosis; Secondly, inhibited β -arrestin recruitment; Thirdly, decreased the stability of the receptor and β -arrestin-2 endosomal complexes and finally inhibited β -arrestin-2-dependent MAPK activation upon agonist stimulation of the V2R. This compound was also used as a tool to determine β -arrestins trafficking function on β 2-adrenergic receptor (β 2AR) recycling.

These results uncover a better understanding of the underlying mechanism regulating β -arrestin in the endosomes as well as the intracellular trafficking modulation of GPCRs. Our findings also reveal a useful tool to investigate the multifunctional aspect of β -arrestins in the cell.

Résumé

Les récepteurs couplés aux protéines G (RCPG) sont l'une des plus grandes familles de récepteurs membranaires et constituent la cible la plus commune pour le développement de drogues à des fins thérapeutiques. Ces récepteurs participent à plusieurs processus physiologiques via la transduction de signaux, généralement induite par les protéines G hétérotrimériques. Suite à leur activation, ces récepteurs à 7 domaines trans-membranaires recrutent l'arrestine afin de procéder à leur endocytose. La β -arrestine associera les RCPG aux différentes composantes de la machinerie d'endocytose incluant la clathrine et la protéine adaptatrice 2 (AP-2), qui permettent aux récepteurs de s'internaliser. Au cours des dernières années, les β -arrestines ont été identifiées comme des protéines de signalisation suite à la liaison de l'agoniste au RCPG; toutefois, le mécanisme qui contrôle les complexes récepteur/ β -arrestine n'est toujours pas élucidé. Nous avons récemment publié une étude démontrant qu'en plus de son rôle d'internalisation, la β -arrestine sert de protéine d'échaffaudage qui lie plusieurs effecteurs de signalisation contenus dans les endosomes, tel que les MAPK. On a donc émis l'hypothèse que l'activité des MAPK est probablement nécessaire pour maintenir le complexe GPCR/ β -arrestine dans les endosomes et qu'en conséquence, elle contribuera à la modification de l'internalisation et du recyclage des récepteurs.

Dans la première étude, nous avons identifié au niveau de la β -arrestine-2 un site de phosphorylation des MAPK (Thr178) suggérant que l'activité des MAPK est effectivement impliquée dans un tel processus. À partir des essais biochimiques et en utilisant la technique FRAP (Recouvrement de la Fluorescence Après Photo-blanchiment) pour déterminer la demi-vie des complexes endosomaux B2R/ β -arrestine-2,

nos résultats ont démontré que le site de MAPK sur l'arrestine joue un rôle important dans l'affinité de l'arrestin à son récepteur. Le rôle du domaine 'hinge' de la β -arrestine-2 a été testé par plusieurs mutations et les résultats démontrent que l'espèce (humaine ou rat) a un impact significatif sur l'affinité de celle-ci envers les RCPG. Cet effet induit en conséquence une régulation différentielle de l'endocytose du récepteur en plus de la réponse signalétique amorcée par l'agoniste. Cette étude met en évidence une corrélation significative entre la signalisation des MAPK et la régulation de la liaison de la β -arrestine aux RCPG dans les endosomes.

En plus du domaine 'hinge' de l'arrestine, plusieurs études de structure ont montré que le 'polar core' et les 'loops' de l'arrestine sont également importants pour l'activation de la β -arrestine-2. En se basant sur la structure cristallographique de la β -arrestine-1, on a pu combiner une approche de criblage virtuel avec des essais de validation cellulaires afin d'identifier des inhibiteurs pharmacologiques sélectifs de la β -arrestine-2. À partir des résultats obtenus, on a identifié un composé, nommé UM0012685, qui d'abord bloque l'endocytose du récepteur vasopressine-V2 (V2R); Deuxièmement inhibe le recrutement de la β -arrestine; Troisièmement diminue la stabilité des complexes endosomaux du récepteur/ β -arrestine-2 et finalement ce composé réduit l'activation des MAPK qui est dépendante de la β -arrestine-2 suite à la stimulation du V2R. Le UM0012685 a aussi été utilisé comme outil pour caractériser la fonction de la β -arrestine-2 sur le recyclage du récepteur β 2-adrénergique (β 2AR).

En résumé, les résultats de cette thèse permettent une meilleure compréhension du mécanisme de régulation des β -arrestines dans les endosomes ainsi que du mécanisme d'action de la désensibilisation et resensibilisation des RCPG. De plus, nous avons

développé un inhibiteur pharmacologique puissant permettant de déterminer la multifonctionnalité de l'arrestine dans le système cellulaire.

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Abbreviation list

Adaptin-associated kinase	AAK
Adaptor protein 2	AP-2
Adenosine tri-phosphate	ATP
Adenylyl cyclase	AC
Angiotensin-converting enzyme	ACE
Angiotensin type 1 receptor	AT1R
Aquaporine-2	AQ-2
Apoptosis signal-regulating kinase 1	ASK1
β 2-adrenergic receptor	β 2AR
β -adrenergic receptor kinases	BARK1
Bcl-2-associated death promoter	BAD
Bradykinin	BK
Bradykinin B2 receptors	B2R
Brain-specific angiogenesis inhibitor 1-3	BAI1-3
Calcium sensing receptor	CaSR
Carboxypeptidase M	CPM
Chemokine receptors type 2 and 5	CCR2/5
Clathrin-coated pits	CCP
Clathrin-coated vesicles	CCV
Cyclic adenosine mono-phosphate	cAMP
c-Jun N-terminal kinase	JNK
C-X-C chemokine receptor type 7	CXCR7
Death-associated protein kinase	DAPK
Diacyl glycerol	DAG
Dishevelled	DVL
Dissociation constant	Kd

Endoplasmic reticulum	ER
Endothelial nitric oxide synthase	eNOS
Epidermal growth factor	EGF
Epidermal growth factor	EGFR
Epithelial Na ⁺ channel	ENaC
Extracellular loops	ECLs
Extracellular signal-regulated kinases	ERK1/2
Fluorescence recovery after photobleaching	FRAP
Fluorescence resonance energy transfer	FRET
Follicle-stimulating hormone	FSH
Gamma-aminobutyric acid	GABA
GPCR autoproteolysis-inducing domain	GAIN
G protein-coupled receptor kinases	GRKs
G protein-coupled receptors	GPCRs
Growth hormone releasing hormone receptor	GHRHR
GTPase-activating protein	GAP
GTPase effector domain	GED
Guanine nucleotide dissociation inhibitor	GDI
Guanine nucleotide exchange factors	GEFs
High molecular weight	HMW
Hormone-binding domain	HBD
Inositol 1,4,5-triphosphate	IP ₃
Insulin growth factor receptor	IGFR
Insulin-like growth factor 1	IGF-1
Intracellular loops	ICLs

kallidin	KD
kallikrein-kinin system	KKS
Leukemia-associated RhoGEF	LARG
Leuteinizing hormone	LH
Lipoprotein-related protein	LRP5/6
Low molecular weight	LMW
Lymphoid blast crisis	Lbc
Lysophosphatidic acid	LPA
MEK-1 dominant negative	MEKDN
Metabotropic glutamate receptors	mGluRs
Metalloproteases	MMPs
Mitogen-activated protein kinase	MAPK
Mouse double minute 2 homolog	Mdm2
Mouse embryo fibroblasts	MEFs
Muscarinic type 2 receptor	M ₂ R
Myosin light chain	MLC
Negative allosteric modulators	NAMs
Nephrogenic Diabetes Insipidus	NDI
Neural precursor cell expressed developmentally down-regulated protein 4	Nedd4
Neurokinin receptor	NK1
Nonclathrin-coated vesicle	NCCV
Nuclear factor-kappa B	NF-κB
p90 ribosomal S6 kinases	RSK
Parathyroid hormone receptor	PTHr
Phosphatidylinositol-4,5-biphosphate	PI(4,5)P ₂
Phosphatidylinositol-3,4,5-triphosphate	PIP ₃

Phosphodiesterase	PDE
Phosphoinositide-3 kinase	PI3K
Phospholipase C	PLC
Platelet-activating factor	PAF
Platelet-derived growth factor receptor	PDGFR
Pleckstrin homology (PH	
Positive allosteric modulators (PAMs)	
Protease-activated receptor 2	PAR2
Protein kinase A/B/C	PKA/B/C
Receptors tyrosine kinases	RTK
Regulator of G protein signaling	RGS
[Sar ⁽¹⁾ , Ile ⁽⁴⁾ , Ile ⁽⁸⁾]-angiotensin II	SII
Seven transmembrane	7TM
Somatostatin receptor subtype 2	SST2
Tumor necrosis factor receptor-associated factor 6	TRAF6
Ubiquitin-specific protease	USP33
Urea transporters	UTA1
Vasopressin	AVP
Vasopressin V2 receptor	V2R

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Authors Contributions

As authorized by the McGill University Faculty of Graduate Studies and Research, this following thesis is presented in a manuscript-based format. It contains two research projects, and listed below are the detailed contributions of each author:

Differential Regulation of Endosomal GPCR/Beta-arrestin Complexes and Trafficking by MAPK. *J Biol Chem.* 2014. Aug 22; 289(34): 23302-17.

Etienne Khoury, Ljiljana Nikolajev, May Simaan, Yoon Namkung, Stéphane A. Laporte.

E. Khoury and S.A. Laporte designed the study and E. Khoury performed the majority of the experiments. L. Nikolajev was involved in FRAP and MAPK experiments. E. Khoury, Y. Namkung and M. Simaan performed the molecular biology. E. Khoury and S.A. Laporte interpreted the data, and wrote the paper.

β -arrestins Inhibitor Discovery by Combined Virtual Screening and Cell-based Assays. *Nature Chemical Biology.* 2014. **Submitted.**

Martin Audet*, **Etienne Khoury***, Wayne Stallaert, Viktoriya Lukasheva, Christian Legouill, Alexandre Beautrait, Stéphane A. Laporte and Michel Bouvier.

In this study, M. Audet helped in constructing the calculation platform, did the virtual screening, designed the rationale and hypothesis of the project and performed all the bench experiments in Michel Bouvier's laboratory. E. Khoury, under the supervision of S.A. Laporte, participated in the design and performed confocal microscopy experiments and MAPK assays (time-dependent and dose-response curves). W. Stallaert

constructed the β arr-2RLucII encoding vector. V. Lukasheva constructed the HA-AT1R encoding vector. C. Legouill built the calculation platform and helped in designing and supervising the experiments. A. Beaudrait helped for the compound mining in the in-house library database. M. Bouvier supervised the work of W. Stellaert, V. Lukasheva, C. Legouill, and A. Beaudrait. M. Audet, E. Khoury, S.A. Laporte and M. Bouvier participated in the writing of the manuscript.

Chapter 1

Introduction

1. The nature of G protein-coupled receptors

1.1. Definition

Every organism must adapt to environmental changes in order to guarantee its survival. The cell membrane, involved in multiple processes including cell adhesion, ion conductivity and cell signaling allow the transition of all extracellular signals to the intracellular milieu. Amongst all proteins embedded on the cell membrane, signal transduction mostly occurs via cell-surface receptors. From these, the G protein-coupled receptors (GPCRs) represent by far the largest, most versatile family of plasma-bound receptors. Comprising approximately 2% of the human genome, GPCRs, also referred to as seven-transmembrane receptors, are involved in almost all physiological responses, including hormonal homeostasis, neurotransmission, taste, vision and smell (Foord, et al. 2005). Therefore, drug development research has focused on studying GPCRs, which are now considered as the most targeted in drug discovery programs (Lagerstrom and Schioth 2008). GPCRs can bind a diverse range of extracellular stimuli, such as biogenic amines (noradrenaline, dopamine, histamine, acetylcholine), lipids (prostaglandins, lysophosphatidic acid (LPA), platelet-activating factor (PAF)), amino acids and ions (glutamate, calcium, gamma-aminobutyric acid (GABA)), peptides and proteins (angiotensin, bradykinin, vasopressin, thrombin, follicle-stimulating hormone (FSH), leuteinizing hormone (LH)), nucleotides, light, odorants and opiates (Marinissen and Gutkind 2001). This wide variety of ligands bind the receptors, trigger conformational rearrangements and activate heterotrimeric G proteins. Upon receptor activation, G proteins stimulate multiple signaling pathways which in turn promote gene transcription

and protein synthesis leading to important biological responses such as cell proliferation, differentiation and survival (Fig. 1).

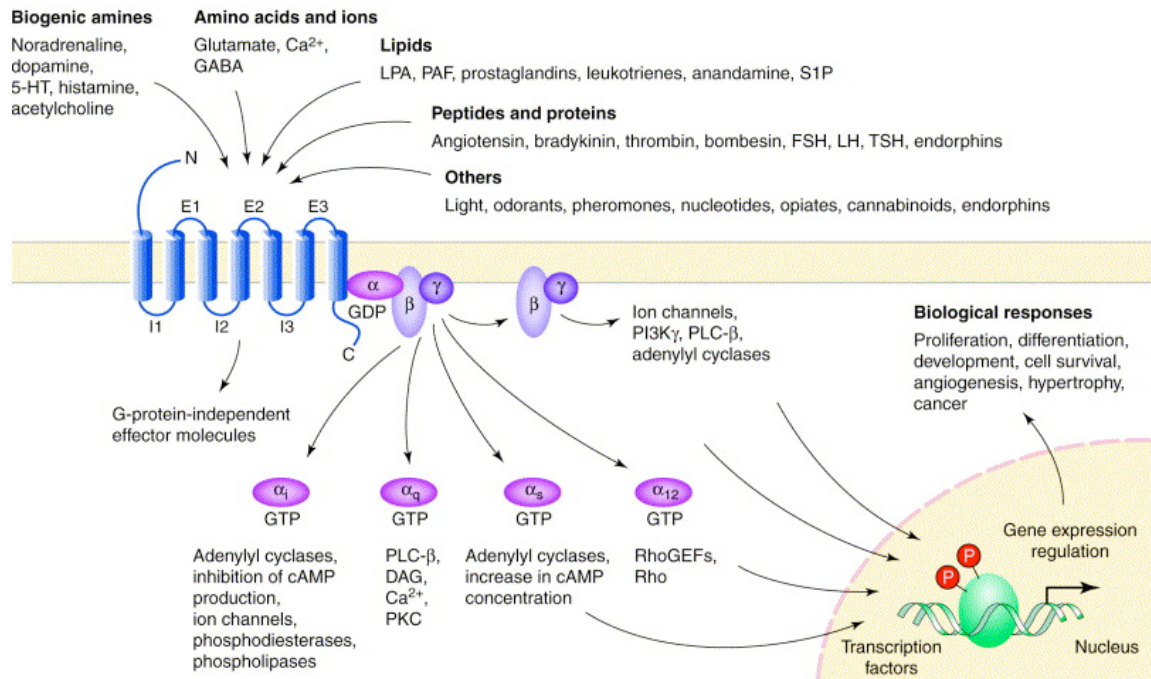


Figure 1. G protein-coupled receptor activation and signaling.

Activated by a wide range of ligands, GPCRs couple to different G proteins subtypes and induce, accordingly, various signaling pathways through the activation of second messengers. Depending on the receptor-mediated signaling cascade, transcription factors will regulate gene expression in the nucleus, and further promote multiple biological responses, including cell proliferation, differentiation, survival, angiogenesis and hypertrophy (Marinissen and Gutkind 2001).

1.2. Structure

The discovery of GPCRs structural features was important to understanding its mechanism of action, and yet many controversies still exist between different versions of receptor activation and G protein recruitment. The first crystal structure of a native GPCR was the vertebrate rhodopsin (Palczewski K 2000). Afterwards, Dr. Kobilka and Dr. Stevens have then reported the crystal structure of the human β 2-adrenergic receptor (β 2AR) bound to its selective antagonist, carazolol (Cherezov, et al. 2007; Rosenbaum, et al. 2007). Several structures of GPCRs have been reported since, such as the adenosine A2 receptor (Liu, et al. 2012; Mustafi and Palczewski 2009) and the C-X-C chemokine receptor type 4 (CXCR4) (Wu, et al. 2010), whereas many other receptors have not been structurally determined yet (*e.g.* angiotensin type 1 receptor (AT1R)). Defining the intermolecular interactions within a receptor was helpful in understanding the link between the structure and the function related to GPCRs.

Structurally, GPCRs are characterized by seven transmembrane (7TM) α -helices, which are connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). The extremities of these hydrophobic transmembrane domains are limited to an extracellular N-terminal and intracellular C-terminal regions implicated in many roles of cell signaling transduction (Fig. 2). The 7 TMs domains, namely TMI to TMVII, represent many kinks, mostly caused by proline residues. Despite their high degree of sequence diversity, these TMs domains surprisingly fit exactly into the plasma membrane layer (Trzaskowski, et al. 2012). The N-terminus region and other residues of the ECLs are targets for post-translational modification such as glycosylation and are essential for the binding of some endogenous ligands to the receptor (Bannert, et al. 2001;

Greenwood, et al. 1997; Khoury, et al. 2014a). Most GPCRs present a well conserved disulfide bond between two cysteines located on the ECL2 and the top extremity of TMIII. This extracellular region is mainly responsible for ligand binding and receptor activation due to its sequence variability and high level of flexibility (Klco, et al. 2006). On the other hand, the C-terminal extremity and the ICLs provide target sites for palmitoylation and/or phosphorylation. Upon agonist binding, this intracellular region undergoes conformational changes and couples to various signaling effectors, such as G proteins and β -arrestins, which further leads to a plethora of receptor-mediated biological responses.

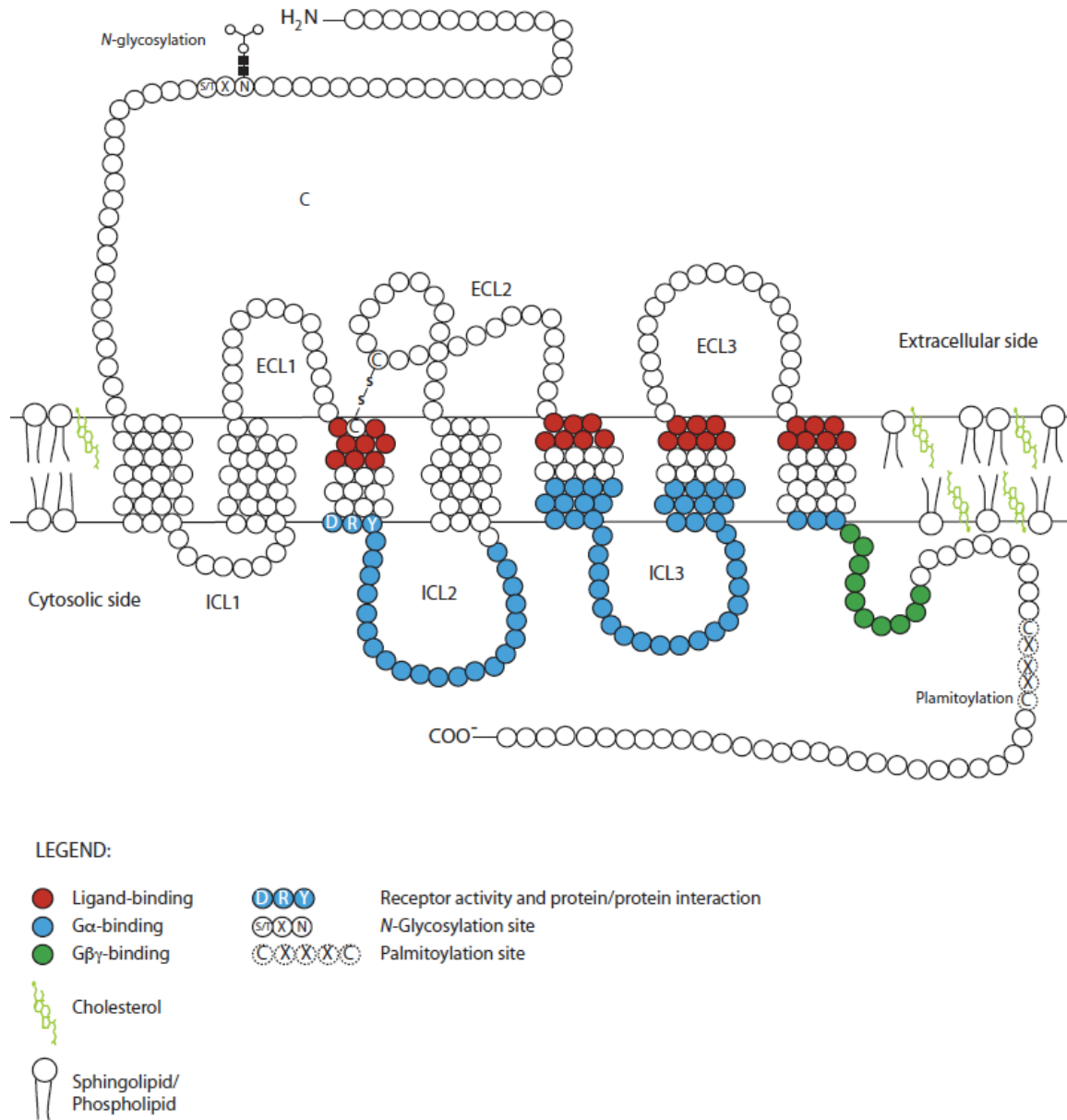


Figure 2. 2-D depiction of a typical G protein-coupled receptor.

GPCRs are seven plasma membrane spanning proteins that consist of three intracellular (ICLs) and three extracellular (ECLs) loops, in addition to an N-terminal domain and a C-terminal tail, highly exposed to several post-translational modifications, such as glycosylation and palmitoylation/phosphorylation, respectively (<http://themedicalbiochemistrypage.org/signal-transduction.php>).

1.3. Classes

Based on their sequence homology and according to the ‘GRAFS’ classification system, GPCRs are divided into five classes: Glutamate receptors, Rhodopsin receptor-like, Adhesion receptors, Frizzled/Taste receptors and peptide-regulated Secretin receptors (Latek, et al. 2012) (Fig. 3).

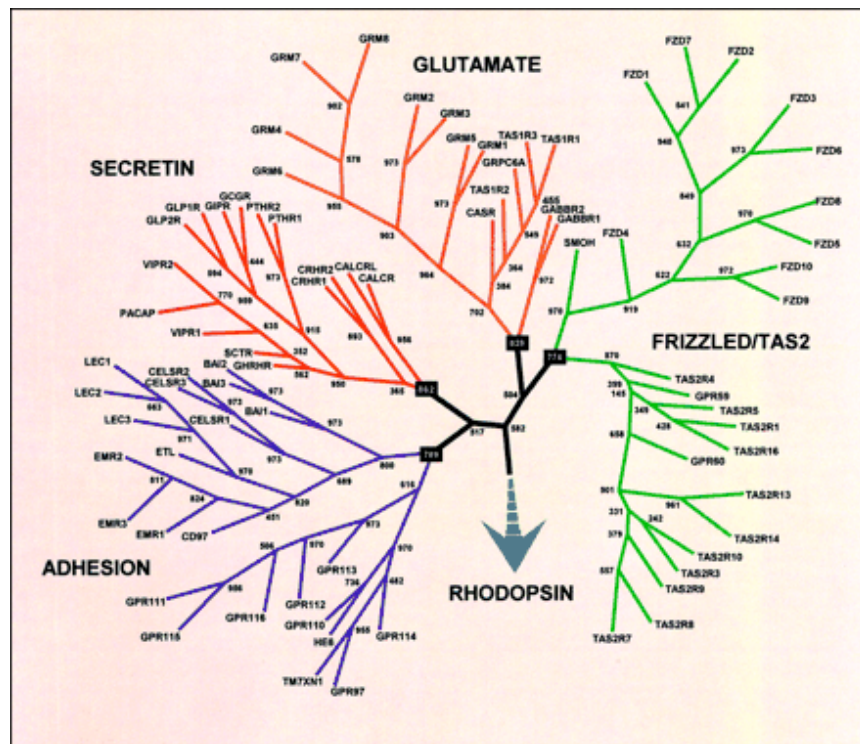


Figure 3. Phylogenetic representation of the G protein-coupled receptors from the human genome (Fredriksson, et al. 2003).

The rhodopsin-like receptor or class A family, which regroups around 700 members is the largest amongst all five classes of receptors. Receptors of the rhodopsin-like family share highly conserved motifs, which includes the Asparagine-Arginine-Tyrosine (DRY) motif located on the cytoplasmic border of TMIII and the NSxxNPxxY on TMVII, both mainly responsible for receptor coupling to G protein (Fig. 4) (Rovati, et al. 2007). Receptors of other families are generally characterized by larger N-terminus structures when compared to rhodopsin-like receptor.

The secretin receptor or class B family shares a N-terminus domain of 60 to 80 amino acids length. Similarly to class A, the class B family receptors comprises many conserved cysteines that are involved in the binding of large peptides, except that the DRY motif does not exist within the TM domains (Fig. 4) (Fredriksson et al. 2003). This class lists a total of 15 receptors including growth hormone releasing hormone receptor (GHRHR), parathyroid hormone receptor (PTHr) and calcitonin receptor.

The glutamate receptor or class C family contains one of the largest N-terminus sequence, and are referred to as the ‘Venus flytrap’ domain. Composed of two lobes, this domain induces structural rearrangements and regulates receptor activation upon ligand binding (Fig. 4) (Kunishima, et al. 2000; Tateyama, et al. 2004). Most studied receptors of this family are the metabotropic glutamate receptors (mGluRs), gamma-amino-butyric acid (GABA_B) receptors, calcium sensing receptor (CaSR) and type 1 taste receptor.

The Adhesion family of receptors contains transmembrane domains very similar to the GPCR classical structures, but have a N-terminal region that shares adhesion-like motifs, such as in the case of epidermal growth factor (EGF) and mucin-like domains, shown to be implicated in cell-cell adhesion (Kristiansen 2004; Stacey, et al. 2000). For

instance, the EGF-TM7 molecules and the F4/80 glycoproteins comprise multiple extracellular EGF domains which encode for several isoforms of receptors mainly expressed in macrophages cell lines (McKnight and Gordon 1998). Another example of adhesion GPCRs is the brain-specific angiogenesis inhibitor 1-3 (BAI1-3), which is highly expressed in the brain. This type of receptor possesses multiple conserved domains such as, hormone-binding domain (HBD), GPCR autoproteolysis-inducing domain (GAIN) and thrombospondin type 1 repeats, and was shown to be involved in several biological processes, mainly cell adhesion (Stephenson, et al. 2014).

Finally, the Frizzled/Taste family of receptors is composed of 10 frizzled receptors and the smoothened receptor. Frizzled receptors contain a cysteine-rich domain on the N-terminal region involved in the binding of the endogenous Wnt hormone (Logan and Nusse 2004). This class of receptors also includes type 2 taste receptors, which is known to have a short N-terminus domain that is not involved in ligand binding (Fredriksson et al. 2003). Other receptor families, not included in the GRAFS classification also exist, such as class D (fungal mating pheromone receptors) and class E (cyclic AMP receptors), but very little is known about their structure-function characteristics.

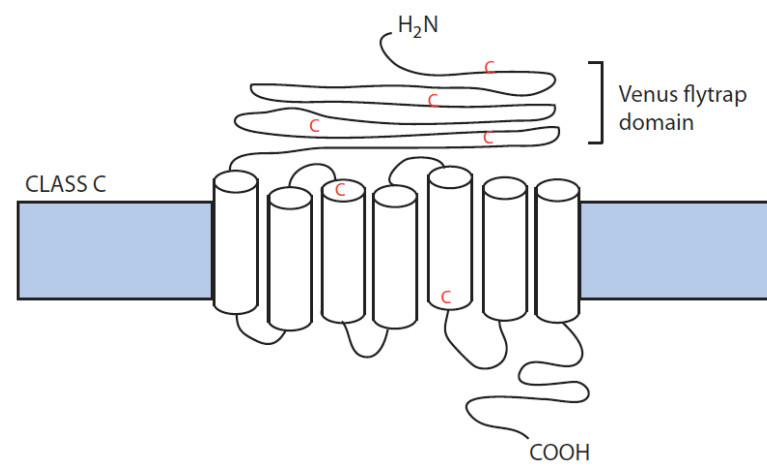
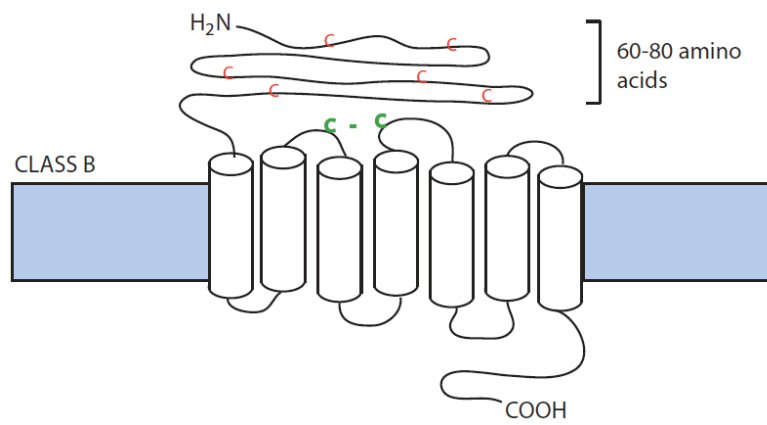
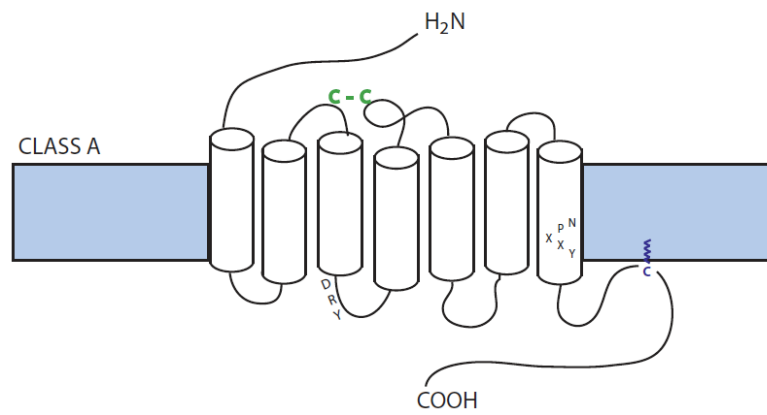


Figure 4. Structural differences between GPCR classes.

Class A receptors share multiple residues in common, including the Cys residues (green) connected by a disulphide bridge between ECL1 and ECL2, the DRY motif from ICL2 and the NPXXY motif from TMVII. Class B receptors share six Cys (red) in their N-terminus domain, in addition to two conserved Cys (green) that form a disulphide bridge. Class C receptors share a long Venus flytrap N-terminal domain that consists on several conserved Cys (red), in addition to many others in the ECLs and TM domains (Adapted from (Rana, et al. 2001)).

2. GPCRs mechanism of action

2.1. Classification of GPCRs ligands

Receptor activation was first described as a single step mechanism that provides a simple biological signal to the cell via a specific precursor, referred to as the endogenous ligand. Therapeutic molecules were then designed to either activate or inhibit receptor signaling responses in order to treat specific disease states. Based on their potencies and efficacies, ligands were classified as agonists, antagonists, and inverse agonists, according to their ability to stabilize the “on” state of receptors allowing the full activation of G proteins such as for agonists, reducing the basal spontaneous coupling to G proteins like in the case of the inverse agonists (*e.g.* maintaining receptor in the “off” state), or inhibiting agonist competitively without changing the equilibrium like for “neutral” antagonists (Fig. 5).

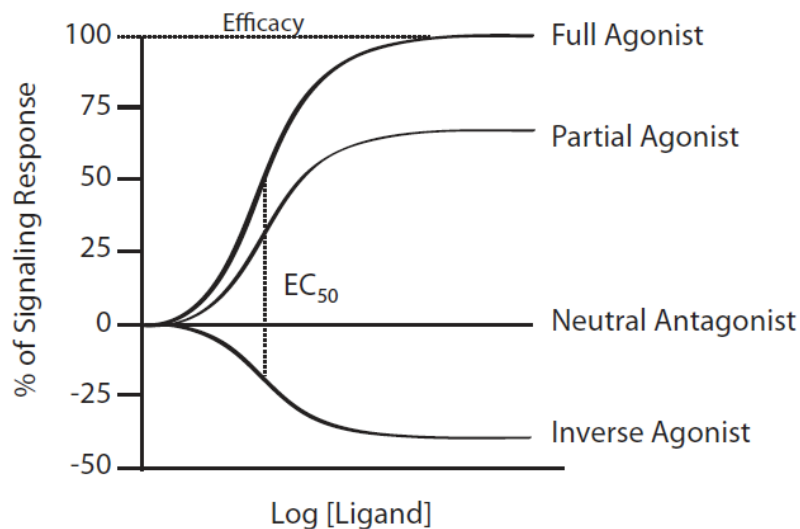


Figure 5. Dose-response curves of different classes of GPCRs ligands.

Ligand efficacy is the maximal effect of a drug on receptor signaling response (*e.g.* 100% for full agonist, less than 100% for a partial agonist), whereas the potency is the concentration of a drug that produces 50% of the full response (EC₅₀).

The endogenous ligand normally interacts with what is defined as the “natural binding pocket” of the receptor, also known as the ‘orthosteric site’. However, other type of ligands, such as ions, small and large molecules (*e.g.* antibodies) and/or protein complexes (*e.g.* receptor dimers and receptor-effector complexes), known as allosteric modulators, also bind to topographically distinct sites from the endogenous ligands. These allosteric ligands modulate hormone binding and/or the intracellular coupling of receptors to their effectors, and affect responses in different ways: They can have differential cooperative effects—negative or positive ones—on the binding of the orthosteric ligand, the “conduit” of the information of the ligand to the effector through

the receptor, and the receptor signaling. Specifically, their effects on signaling have been mainly divided into two categories: positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs). This concept will not be further discussed here but can be reviewed in Khoury et al. 2014 (Khoury et al. 2014a).

2.2. GPCR activation

GPCR activation was first described by a classical two-state model, where receptors exist in equilibrium between active (*e.g.* G protein-coupled: the “on” state) and inactive states (*e.g.* G protein-uncoupled: the “off” state), and where extracellular stimuli, such as hormones, neurotransmitters, peptides and amino acids, shift this equilibrium from one state to the other (Marinissen and Gutkind 2001). However, several new lines of evidence now support an alternative multi-state model, where GPCRs can adopt multiple conformations, including active, inactive and other intermediate ones. In such multi-state model, it is also inferred that ligands have the propensity of stabilizing a unique conformation leading to a specific signaling response, which may or may not always totally mimic the one induced by a natural ligand of reference. These ligands can stabilize a “hybrid” receptor conformation that mimics the “on” conformation with respect to engaging one signaling pathway, while at the same time mimicking the “off” conformation for another signaling pathway that is normally activated by an agonist of reference. Such mode of ligand-mediated differential signaling is commonly referred to as “functional selectivity” or “ligand-biased signaling” (Luttrell 2013; Rajagopal, et al. 2010; Violin and Lefkowitz 2007). Moreover, it is now well accepted that orthosteric ligands, allosteric modulators and receptor domains that participate in ligand and/or

effector binding, can also be targeted to bias signaling (Khoury et al. 2014a). Biased signaling will be more expanded in the section of 'Role of β -arrestins in signaling'.

2.3. Heterotrimeric G proteins

2.3.1. G protein activation

The ternary complex model of the agonist/receptor/G protein was first described by Dr. Lean (De Lean, et al. 1980). Using radio-labeled receptor ligands, the receptor was shown to have a high and a low affinity binding state to its ligand. The affinity of the agonist to its orthosteric site was dependent on the activity of a guanine nucleotide protein, which was defined as the G protein. Because the administration of GTP affected the interaction between the agonist and its receptor, the G protein was suspected to act as an allosteric ligand and bind the receptor from its intracellular region.

The heterotrimeric G proteins are composed of three subunits: the nucleotide binding α -subunit ($G\alpha$) and the $G\beta$ and $G\gamma$ subunits, which form stable dimers ($G\beta\gamma$). At basal state, the $G\alpha$ -subunit remains bound to GDP and tightly associated to $G\beta\gamma$ dimers. Upon agonist binding, the receptor undergoes conformational changes, binds the GDP-bound $G\alpha$ protein, which will exchange the GDP for GTP and leads to subsequent dissociation of the newly formed GTP-bound $G\alpha$ from $G\beta\gamma$ subunits (Latek et al. 2012). G protein subunits will then activate multiple second messengers, which in turn will induce various downstream signaling pathways. However, the $G\alpha$ subunit also contains a low intrinsic GTPase activity, which will terminate the receptor signals by hydrolyzing the GTP to GDP and re-associating once again the GDP-bound $G\alpha$ back to $G\beta\gamma$ subunits (Northup, et al. 1983). This GTPase activity is enhanced by GTPase-activating proteins

(GAPs), which are referred to as the regulator of G protein signaling (RGS) for GPCRs. This dynamic cycle of GTP/GDP exchange during GPCR activation is shown in figure 6.

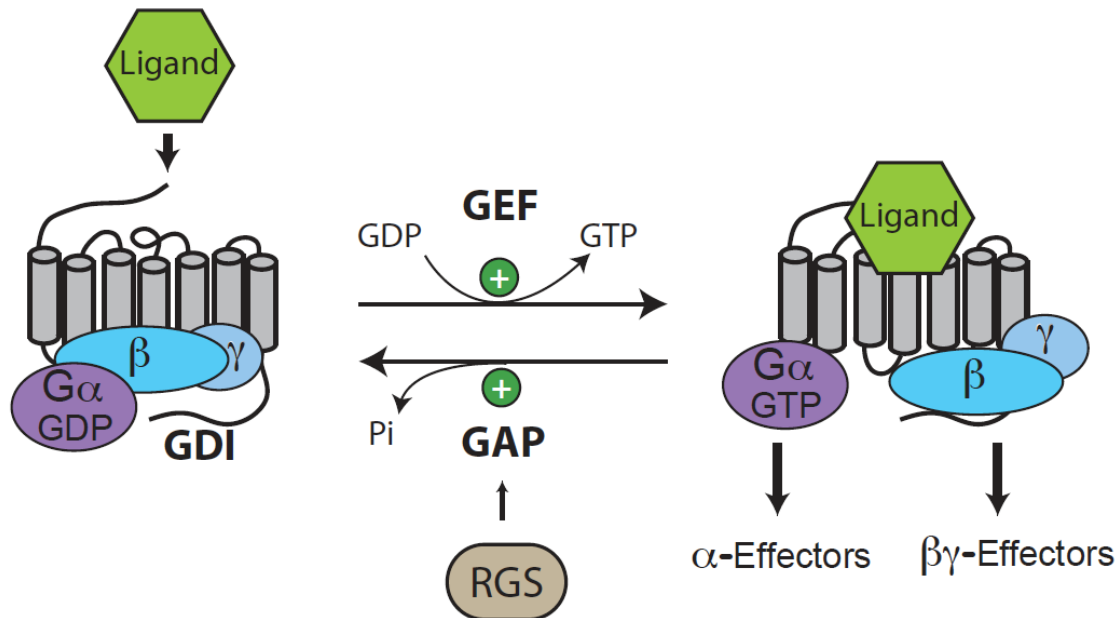


Figure 6. The guanine nucleotide cycle during G protein activation.

At basal state, $G\beta\gamma$ subunits, associated with $G\alpha$, serve as guanine nucleotide dissociation inhibitor (GDI) preventing the release of GDP from the $G\alpha$ protein. Upon ligand binding, the activation of guanine nucleotide exchange factors (GEFs) induces the exchange of GDP to GTP and promotes $G\alpha$ and $G\beta\gamma$ dissociation, which will lead to the activation of downstream effectors. Receptor signal is then terminated by the regulator of G protein signaling (RGS), a GTPase-accelerating protein (GAP) for $G\alpha$, which promotes the GDP-bound $G\alpha$ form by increasing the GTP hydrolysis rate (Adapted from (Siderovski and Willard 2005)).

Several lines of evidence have questioned the classical model of GPCR activation, and many studies proposed an alternative mechanism where G proteins are pre-coupled to the receptor at basal state. An example of this is a study using fluorescence resonance energy transfer (FRET) to monitor the conformational rearrangements at the interface between the α_2 -adrenergic receptor and G proteins, and between the G proteins subunits. In untreated conditions, a low basal FRET signal was detected between the receptor and $G\alpha_i$ and between the $G\alpha_i$ and $G\beta\gamma$. Moreover, results also demonstrated that agonist binding triggers conformational reorganization of pre-assembled receptor/G protein complexes, which leads to the opening, but not dissociation, of the $G\alpha/G\beta\gamma$ interface (Gales, et al. 2006). Another example of a ‘pre-coupling’ model is the dark-adapted rhodopsin, which binds at basal state the heterotrimeric transducin (Gt), with a dissociation constant (Kd) of 64 nM, and the affinity increases to 0.7 nM under light activation (Alves, et al. 2005). However, the existence of such pre-coupling complex was not obvious to other similar studies, which in return concluded that the kinetics of G protein/receptor interactions could be highly influenced by the concentrations of both elements in the cell, in a way that the overexpression of whether the G protein and/or the receptor would force the complex to form even without ligand treatment (Hein, et al. 2005). Until today, these two conceptual views are still under debate.

2.3.2. G protein classes and downstream signaling effectors

The activation of cell signaling effectors is mainly dependent on the G protein subtypes. The α -subunit is divided into four groups, namely $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ (Gilman 1987) and we can count up to five different $G\beta$ ($G\beta_{1-5}$) and twelve $G\gamma$ ($G\gamma_{1-12}$)

subunits (Cabrera-Vera, et al. 2003).

Isoproterenol-activated β -adrenergic receptors are one of the first GPCRs known to couple to $G\alpha_s$ (Brandt, et al. 1983; Cerione, et al. 1984). Briefly, the ubiquitously expressed $G\alpha_s$ protein activates adenylyl cyclase (AC), whose main function is to catalyze the conversion of the adenosine tri-phosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Beavo and Brunton 2002). Increased levels of intracellular cAMP activates protein kinase A (PKA), which in turn will phosphorylate a wide range of proteins, including GPCRs, protein kinases (e.g. mitogen-activated protein kinase (MAPK)), phosphatases and transcription factors and further promote multiple biological responses (Smith, et al. 2011). Contrarily to the $G\alpha_s$ function, members of the $G\alpha_{i/o}$ family (G_{α_i} , $G\alpha_{i1}$, $G\alpha_{i2}$, G_{α_o1} and G_{α_o2}) inhibit AC and decrease cAMP levels. This family of G proteins is widely expressed, and its high level of expression results in the release of high amounts of $\beta\gamma$ complexes. Therefore, studies suggested that the activation of the $G\alpha_{i/o}$ is mainly implicated in the $\beta\gamma$ -mediated signaling pathways (Clapham and Neer 1997). On the other hand, another homolog of the $G\alpha_o$, the rod-transducin (G_t), known to couple to rhodopsin, binds and activates the cGMP-phosphodiesterase (PDE) by blocking the inhibitory γ -subunit of the retinal type 6 PDE (PDE6). The activation of PDE downregulates the cGMP-activated potassium channels, thus leading to hyperpolarization of retinal ganglion cells (Arshavsky, et al. 2002; Latek et al. 2012).

The $G\alpha_{q/11}$ protein activates the β -isoforms of phosphoinositide-specific phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), a membrane phospholipid, to inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG) (Rhee 2001). Intracellular IP_3 binds and activates the IP_3

receptor (IP₃R), which is a Ca²⁺-release channel mainly located on the endoplasmic reticulum (ER) surface and responsible for controlling the Ca²⁺ release in the cytoplasm (Vanderheyden, et al. 2009). The elevated concentration of the calcium ion (Ca²⁺) in the cytosol triggers various cellular events, such as gene transcription, cell proliferation and muscle contraction (Berridge 1993). In synergy with increased calcium concentrations, the DAG activates protein kinase C (PKC), a phospholipid-dependent serine/threonine kinase involved in a wide range of cell signaling pathways leading to cell growth, migration and apoptosis (Newton 2010; Steinberg 2008).

By using sequence alignments between G protein subtypes, G α_{12} and G α_{13} protein members were discovered last of all three G α proteins cited above (Strathmann and Simon 1991). G $\alpha_{12/13}$ -coupled receptors mainly activate RhoGEFs, which are divided into four family members: p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG) and lymphoid blast crisis (Lbc)-RhoGEF (Dutt, et al. 2004; Fukuhara, et al. 2000, 2001; Kurose 2003). RhoGEFs catalyze the conversion of GDP to GTP-bound G $\alpha_{12/13}$ and activate small monomeric GTPase RhoA (Sah, et al. 2000). RhoA was shown to be associated with ROCK activation and other signaling effectors. Once activated, ROCK inhibits the myosin light chain (MLC) phosphatase, which leads to the phosphorylation of MLC and promotes cell contraction (Riento and Ridley 2003). Ubiquitously expressed, G $\alpha_{12/13}$ protein plays important roles during pathological conditions, such as in cardiovascular diseases, bronchial asthma and cancer (Siehler 2009). Therefore, G $\alpha_{12/13}$ -induced signaling pathways are considered as potential targets for such GPCR-related diseases. However, research advances on this class of G proteins has been very limited because no specific

inhibitors of the $G\alpha_{12/13}$ have yet been developed.

The $G\beta\gamma$ heterodimers, considered as a single entity, are also known to play an important role in GPCRs signaling. One of the first signaling roles identified for $G\beta\gamma$ was shown by a study using purified $G\beta\gamma$ subunits from bovine cerebral cortex and showing that such complex is responsible of activating cardiac muscarinic-gated potassium channels (Logothetis, et al. 1987). Other than acting as a negative regulator of the $G\alpha$, $G\beta\gamma$ subunits are now known to regulate multiple signaling effectors, such as AC isoforms (Taussig, et al. 1994), phosphoinositide-3 kinase (PI3K) (Stephens, et al. 1994), PLC β (Philip, et al. 2007) and voltage-gated calcium channels (Zamponi, et al. 1997). $G\beta\gamma$ signaling pathways will not be expanded further here as it has been well covered by many reviews (Dupre, et al. 2009; Milligan and Kostenis 2006; Oldham and Hamm 2008). A summary of all G protein subtypes with downstream signaling effectors are presented in table 1.

Table 1. Heterotrimeric G protein subtypes and downstream signaling effectors.

G-protein class	G-protein subunits	Effectors	Regulation of effector	References
Gs	G α s	AC1-9 c-Src RGS-PX1 Tubulin	Stimulation Stimulation Stimulation Stimulation of GTPase activity	Pierce et al., 2002; Cabrera-Vera et al.,2003
Gi	Golf G α tr G α tc G α g G α i1 – 3, G α o	ACS PDE6 PDE6 PDE AC5, AC6 c-Src RAP1GAPII Ca ²⁺ channels G-protein-activated inwardly rectifying K ⁺ channel (GIRK) channels Tubulin G-protein-regulated inducer of neurite outgrowth (GRIN)	Stimulation Stimulation Stimulation Stimulation Inhibition Stimulation Sequestration of RAP1GAP Inhibition (G α o) Stimulation (G α i) Stimulation of GTPase activity Unknown	Mochizuki et al., 1999; Pierce et al., 2002; Cabrera-Vera et al.,2003
Gq	G α q, 11, 14, 15/16	1 and GRIN2 PLC β 1 – 4 Leukemia-associated-RhoGEF (LARG-RhoGEF) GRK2, GRK3 Bruton's tyrosine kinase	Stimulation Stimulation (indirect activation of Rho) Stimulation Stimulation (indirect stimulation of p38 MAPK)	Pierce et al., 2002; Cabrera-Vera et al.,2003 Willets et al., 2003 Jiang et al., 1998;
G12	G α 12, G α 13	P115-RhoGEF, PDZ RhoGEF, LARG-RhoGEF GTPase-activating protein of ras (GAP1m) E-cadherin	Stimulation (indirect activation of Rho and stress fiber formation) Stimulation (indirect activation of ras) Stimulation (stimulation of β -catenin release)	Pierce et al., 2002
	G $\beta\gamma$	PI3K γ , PI3K β AC2 ^a , AC4 ^a , AC7 AC1 PLC β 1 – 3 GRK2, GRK3 GIRK1, GIRK2, and GIRK4 ATP-sensitive K ⁺ channels Ca ²⁺ channels Small GTP binding proteins (Rho and Rac) Bruton's tyrosine kinase Tsk tyrosine kinase Protein kinase D Calmodulin Tubulin Dynamin I Shc Raf1 protein kinase Ras guanine nucleotide releasing factor/ CDC25Mm KSR-1	Stimulation Stimulation Inhibition Stimulation Recruitment to plasma membrane Stimulation Stimulation Inhibition Recruitment to plasma membrane Stimulation Stimulation Stimulation Inhibition of calmodulin kinase Increased GTPase activity Increased GTPase activity Stimulation (indirect activation of MAPK) Sequestration of G $\beta\gamma$ Stimulation (indirect activation of ras) Sequestration of G $\beta\gamma$	Pierce et al., 2002; Cabrera-Vera et al.,2003 Willets et al., 2003

^a AC2 and AC4 are synergistically activated by α s and $\beta\gamma$.

AC, adenylyl cyclase; PDE, phosphodiesterase; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; GRK, G protein-regulated kinase; RhoGEF, Rho guanine nucleotide exchange factor; KSR, kinase suppressor of Ras (Modified from (Kristiansen 2004)).

2.4. GPCR trafficking: desensitization and internalization.

The heptahelical receptors exist in an equilibrium state, balancing between cell surface, intracellular compartments, protein synthesis and degradation. GPCRs responsiveness is mainly controlled by two major elements, either the signaling efficiency induced from the receptor itself or the level of receptors at the plasma membrane. In fact, intracellular signaling of GPCRs is mediated by three distinct mode of regulation: (1) Desensitization, where receptors undergo a negative feed-back process that will lead to signaling inactivation; (2) Internalization, which causes the physical removal of the receptor from the plasma membrane by endocytosis; (3) Degradation/Resensitization, where the receptor is either downregulated by lysosomal control or recycled back to the plasma membrane to reset its signaling activities (Fig. 7) (Gainetdinov, et al. 2004; Tian, et al. 2014).

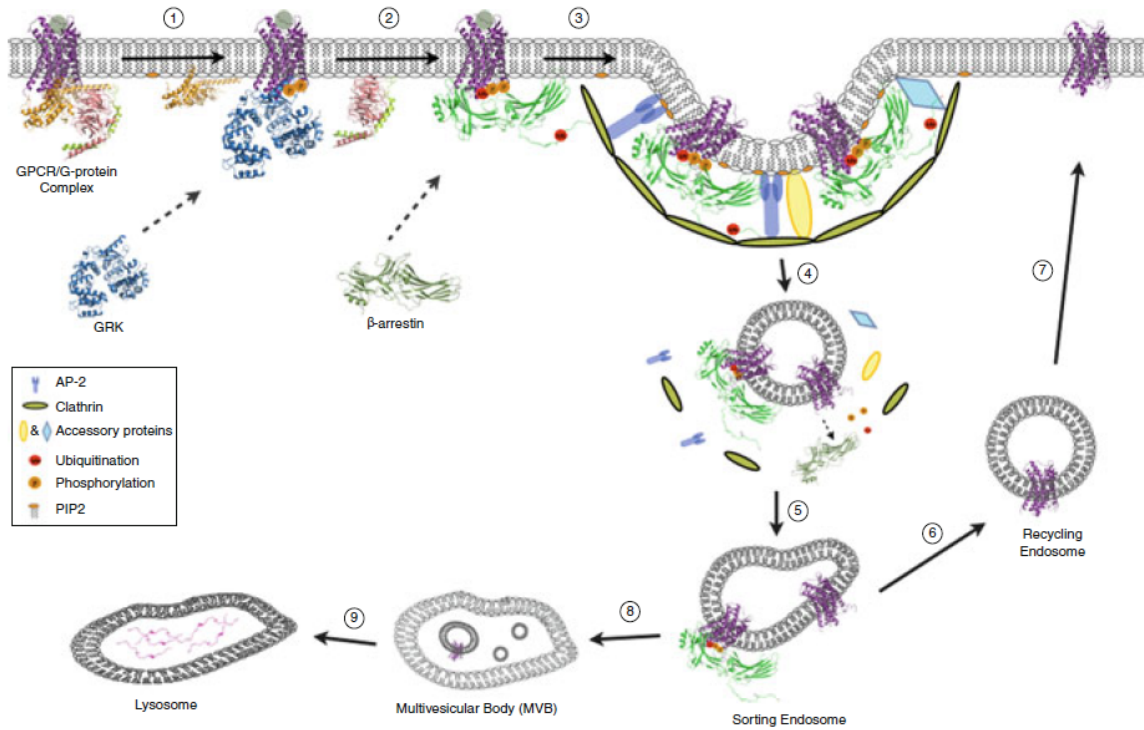


Figure 7. Receptor desensitization and internalization.

Ligand binding to GPCR promotes G protein-kinase-dependent receptor phosphorylation (1), the binding of β -arrestin to the receptor (2) and the recruitment of the endocytic machinery (3) leading to a clathrin-dependent internalization of the complex (4). Receptor/ β -arrestin complexes traffic to sorting endosomes (5) which will drive the receptor into two distinct pathways: (6,7) Receptor recycling or (8,9) Lysosomal degradation of the receptor (Tian et al. 2014).

2.4.1. Receptor desensitization

GPCR desensitization involves the uncoupling of the G protein from the transmembrane receptor, which presumably stops ligand-mediated receptor activation and consequently terminates all receptor downstream signaling pathways. After ligand binding, GPCRs are phosphorylated on serine/threonine residues located within the intracellular domains, more specifically on the third intracellular loop and the C-terminal tail. This phosphorylation is mediated by two types of kinases: the intracellular second messengers-regulated protein kinases, such as PKA and PKC and the G protein-coupled receptor kinases (GRKs). The second messengers-regulated protein kinases are capable of phosphorylating the receptor, whether in 'active' or 'inactive' state, and this process is referred to as 'heterologous' desensitization (Bouvier, et al. 1989; Freedman and Lefkowitz 1996). Moreover, in some cases, such protein kinases do not only desensitize receptor signaling but may also switch the G protein coupling and shift the signaling from one pathway to another. For example, it has been shown that the phosphorylation of $\beta 1AR$ by PKA induces $G\alpha_i$ coupling to the receptor instead of the $G\alpha_s$ that is normally activated by the agonist-bound receptor (Martin, et al. 2004). On the other hand, GRKs phosphorylate the receptor only when occupied by its ligand, leading to 'homologous' desensitization. Based on sequence homology and functional similarities, GRKs were divided into three subfamilies: GRK1/7; GRK2/3 and GRK4/5/6 (Benovic, et al. 1987). Most of these GRK members are ubiquitously expressed throughout the body. GRK 2 and 3, also referred to as BARK1 and 2 (β -adrenergic receptor kinases), were first identified as kinases involved in the phosphorylation of the $\beta 2AR$ (Benovic, et al. 1986). GRK 1 and 7 are primarily implicated in visual functions and their expression is only

limited to rod and cone photoreceptors. GRK 4 is predominantly expressed in the testis, GRK 5 in the heart and GRK 6 in the brain (Gainetdinov, et al. 2000; Pitcher, et al. 1998). Structurally, all GRKs are composed of three functional domains: The unique N-terminal region, followed by the regulator of G protein signaling (RGS) homology domain (RH) (Siderovski, et al. 1996); The Ser/Thr protein kinase domain (KD); and the C-terminal region responsible for GRKs membrane targeting (Gurevich, et al. 2012). The C-terminal domain varies between GRK subtypes, as it either contains a short prenylation sequence (GRK 1 and 7); pleckstrin homology (PH) domains which bind the G $\beta\gamma$ proteins (GRK 2 and 3); multiple palmitoylation sites (GRK 4 and 6); or phospholipid-binding domains (GRK 5). While GRKs are not sufficient to fully prevent GPCR/G protein coupling, they also promote high affinity binding sites on the receptor, leading to the recruitment of β -arrestins which in turn will guarantee the removal of the receptor from the cell surface. This latter process, called internalization, will be discussed in the following section.

2.4.2. Receptor internalization

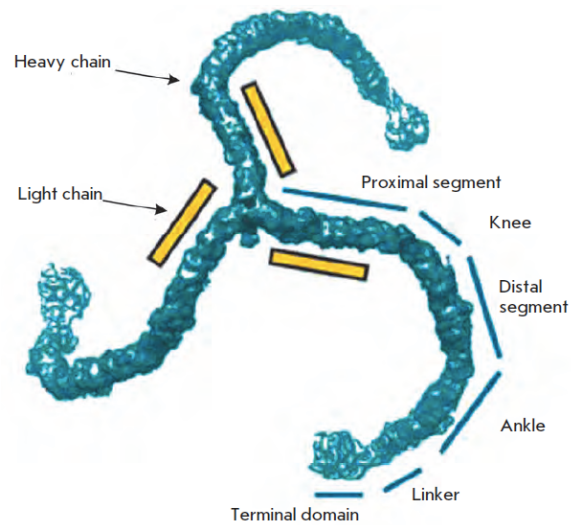
Following receptor desensitization, GPCRs recruit the endocytic machinery to the plasma membrane, which allows the receptor to internalize into endosomal compartments. However, receptor desensitization and internalization are two distinct events. Unlike the β 2AR, the α 2a-adrenergic receptor has been shown to be desensitized upon ligand treatment, but does not internalize into intracellular endosomes (Daunt, et al. 1997). In contrast, other receptors, such as the thromboxane β receptor, undergo constitutive internalization in a ligand-independent manner (Parent, et al. 2001). Amongst

multiple pathways of internalization, the most common and best-characterized process of receptors' sequestration from the cell surface is the clathrin-mediated endocytosis.

2.4.2.1. Clathrin Coated Vesicles-mediated internalization

In 1975, Clathrin-coated vesicles (CCV) were first isolated and purified from pig brain (Pearse 1975). The clathrin, which is the most abundant protein amongst these vesicles, resembles to a triskelion and comprises three heavy and three light chains (Fotin, et al. 2004). The proximal domains of the heavy chains are associated to the light chains and mainly responsible for the formation of ordered clathrin lattice (*e.g.* 'clathrates') (Fig. 8) (Popova, et al. 2013). Structurally, light chains consist of three distinct domains: a conserved C-terminal region, a central portion of α -helical structure and a N-terminal domain that contains two conserved motifs identified as binding sites for adaptor proteins, such as β -adaptin, β -arrestin and amphiphysins 1/2 (ter Haar, et al. 2000).

(A)



(B)



Figure 8. Schematic representation of the clathrin structure.

(A) Triskelion structure of the clathrin. The N-terminus is identified as the ‘terminal domain’ and the center of the molecule, close to the ‘proximal segment’, is the C-terminal domain. (B) The Hexagonal barrel representation of clathrin self-association (Popova et al. 2013).

Receptor internalization is mainly triggered by two essential adaptor proteins; β -arrestin and adaptor protein 2 (AP-2) (Kirchhausen, et al. 1997). Upon ligand binding, GPCRs are phosphorylated by GRKs, which further leads to the recruitment of β -arrestin to the activated receptor. β -arrestins play an important role in GPCR internalization, as their interaction with desensitized receptors is essential for the binding of clathrin and AP-2 (Laporte, et al. 2002; Santini, et al. 2000). Beta-arrestins structure and function will be discussed in detail in the following sections.

2.4.2.1.1. Adaptor protein complex

The AP-2 complex was the first protein to be identified as clathrin adaptor, characterized by its ability to promote the assembly of the clathrin lattice during the internalization process. Based on sequence homology and cellular functions, adaptor proteins are divided into four different classes, AP-1, AP-2, AP-3 and AP-4. The best two characterized adaptors are the AP-1 which binds to the trans-Golgi network and the AP-2 complex generally recruited to the plasma membrane (Schmid 1997). These tetrameric adaptor complexes comprises four subunits: two of them with high molecular weight (γ - and β_1 -adaptins for AP-1; α - and β_2 -adaptins for AP-2), a medium size subunit (μ_1 for AP-1; μ_2 for AP-2) and a low molecular weight subunit (σ_1 for AP-1; σ_2 for AP-2) (Fig. 9) (Popova et al. 2013).

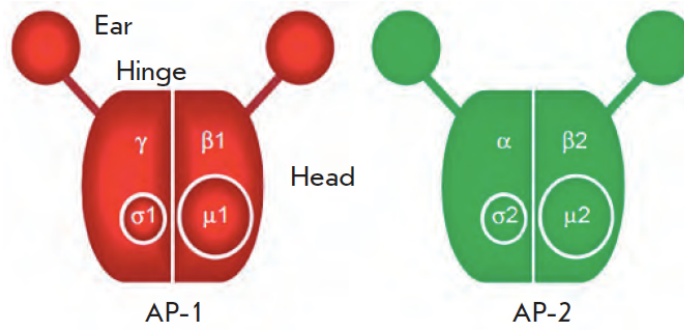


Figure 9. The adaptor protein complexes (Popova et al. 2013).

The heterotetrameric AP-2 complex plays an important role in the formation of CCVs by associating the desensitized receptor to the clathrin lattice. The α -subunit of AP-2 will first bind the phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) located at the plasma membrane and this interaction will be tightly maintained through the phosphorylation of the μ_2 subunit by the adaptin-associated kinase (AAK) (Honing, et al. 2005; Ricotta, et al. 2002). Moreover, α -subunit also binds clathrin and other accessory proteins such as dynamin (Goodman and Keen 1995) and Epsin15 (Benmerah, et al. 1996). Other than binding to β -arrestins, the β_2 -subunit of AP-2 also interacts with clathrin through a specific motif named the “clathrin box” (L ϕ X ϕ [D/E], where ϕ stands for any large hydrophobic amino acid), located in the hinge domain of the β_2 -adaptin.

2.4.2.1.2. Dynamin

After the assembly of the endocytic machinery, clathrin-coated vesicles (CCVs), also known as clathrin-coated pits (CCPs) are detached from the plasma membrane and released into the cytosol for further trafficking events. Dynamin, a 98 KDa GTPase located on the neck of the vesicles, is responsible for pinching the endocytic pits off the

surface membrane via GTP hydrolysis. Dynamin was first discovered in 1987 as a microtubule-binding protein (Paschal, et al. 1987) and its role in endocytosis was then revealed when the *Drosophila* gene *shibire*, an homologue of dynamin, was sequenced (van der Blik and Meyerowitz 1991). In fact, when exposed to a non-permissive temperature, the termini nerve of *shibire* fly (a temperature-sensitive *Drosophila* mutant) showed a decrease in the endocytosis process (Kosaka and Ikeda 1983). Structurally, dynamin is composed of five domains: a N-terminal region that contains a GTPase domain; a middle domain; a GTPase effector domain (GED) involved in the self-assembly of dynamin; a PH domain which mediates the binding of dynamin to lipids (e.g. PIP₂) and facilitates its localization to the plasma membrane; and a proline-rich domain at C-terminal region which contains multiple SH3-binding sites important in protein-protein interactions (e.g. amphiphysin and endophilin) (Chen, et al. 2004b; Farsad, et al. 2001; Takei, et al. 1999). Amongst these domains, the GED has been characterized as the main promoter of the GTPase activity, therefore, acting as a GAP for dynamin (Muhlberg, et al. 1997). More specifically, it was shown that several residues of the GED are involved in the dynamin function, such as K694, responsible for the structural rearrangement of the dynamin assembly, R725, mainly implicated in the catalytic activity (Sever, et al. 1999), and K44 involved in the GTP binding where the substitution of such residue to an alanine (K44A) causes inhibition of receptor-mediated internalization (Chen et al. 2004b; Herskovits, et al. 1993). The detachment of the CCVs from the plasma membrane is due to conformational changes of the dynamin resulting in the constriction of the vesicles' neck from the lipid layer (Ferguson and De Camilli 2012). After dynamin assembly and complete detachment of CCVs from the plasma membrane, clathrin undergo

depolymerization and immediately dissociates from the surface of the vesicles so that the CCV can fuse with early endosomes in order to deliver the receptor to its final destination. According to the endosomal scaffolding complexes, receptors can be directed to various trafficking vesicles, such as recycling endosomes or late endosomes/lysosomes.

2.4.2.2. Caveolae/lipid raft pathway

Besides CCVs-mediated internalization, GPCRs also internalize via a different endocytic process, namely the caveolae/lipid raft pathway where receptors are able to internalize even in the absence of β -arrestin or clathrin. For example, studies have shown that glucagon receptor (Krillov, et al. 2008), CXCR7 (Mahabaleshwar, et al. 2012) and somatostatin receptor subtype 2 (SST2) (Brasselet, et al. 2002) internalize in a β -arrestin-independent manner. Other receptors were also shown to internalize through the caveolae pathway, such as chemokine receptors type 2 and 5 (CCR2/5) (Dzenko, et al. 2001; Mueller, et al. 2002), adenosine A1 receptor (Escriche, et al. 2003) and cannabinoid CB1 receptor (Keren and Sarne 2003). Similarly to the clathrin-mediated endocytosis, the caveolae-dependent internalization still uses the dynamin for vesicles removal from the plasma membrane, however does not direct receptors into endosomal trafficking pathways but rather target them to the endoplasmic reticulum, which is for example the case of the autocrine motility factor receptor (Benlimame, et al. 1998). The endocytic sorting of GPCRs can vary from a receptor to another depending on the agonist type, the GRK-mediated receptor phosphorylation and the availability of the endogenous proteins involved in the internalization process. The synergy of action between these elements can favor the receptor to undergo an endocytic route versus another or even internalize via

both. For instance, in the case of the endothelin receptors, a study showed that the inhibition of the caveolae process forced the receptor to switch to clathrin-mediated internalization (Okamoto, et al. 2000).

2.4.2.3. NonClathrin-Coated Vesicle-mediated internalization

Finally, GPCRs can also use a nonclathrin-coated vesicle (NCCV) pathway to undergo internalization. This alternative endocytic route was shown to exist only when both caveolae- and clathrin-mediated internalization were inhibited. The acetylcholine muscarinic type 2 receptor (M₂R) (Roseberry and Hosey 2001) and the secretin receptor (Walker, et al. 1999) are examples of receptors known to internalize through the NCCV pathway. While differences between those endocytic routes have been identified for several GPCRs, the exact mechanism underlying receptor desensitization and internalization is still poorly characterized.

3. The Kallikrein-Kinin system

In the early 1900s, the kallikrein-kinin system (KKS) was characterized as a hypotensive factor when first extracted from urine. Kallikrein, derived from *kallikréas*, meaning pancreas in Greek, was named because of its abundant expression in the pancreas (Bhoola, et al. 1992). Research studies have then demonstrated that kallikrein produces an active substance, called kallidin (KD), which was derived from an inactive precursor, named kininogen. Meanwhile, a different research group investigated an identical peptide found in snake venoms, and was then referred to as bradykinin (BK) because of its significant effect on lowering blood pressure (Bhoola et al. 1992). Over the

years, a better understanding of the metabolic kallikrein-kinin cascade was established, and several studies showed that BK is produced from the plasma KKS through plasma kallikrein acting on high molecular weight (HMW) kininogen, whereas the KD is synthesized from the tissue-specific KKS via tissue kallikrein acting on low molecular weight (LMW) kininogen. These active peptides are then cleaved by either membrane-bound carboxypeptidase M (CPM) or soluble plasma carboxypeptidase N (deletion of the C-terminal Arg residue) and form des-Arg⁹-BK and des-Arg¹⁰-KD, respectively (Fig. 10) (Kuhr, et al. 2010; Skidgel and Erdos 1998; Skidgel, et al. 2006).

3.1. The Bradykinin receptors

The nonapeptide BK (Arg¹-Pro²-Pro³-Gly⁴-Phe⁵-Ser⁶-Pro⁷-Phe⁸-Arg⁹) and the decapeptide KD (Lys⁰-BK) mediate their cellular and physiological actions via two subtypes of GPCRs. The BK and KD bind the bradykinin B2 receptors (B2R), which are constitutively expressed in various cell types, whereas des-Arg¹⁰-KD and des-Arg⁹-BK interact with bradykinin B1 receptors (B1R), which maintain a lower level of expression under physiological conditions, but rapidly respond to inflammatory stimuli and tissue injury (Bhoola et al. 1992; Schanstra, et al. 1998).

In endothelial cells, kinin-mediated activation of B2R generally stimulates the G $\alpha_{q/11}$ signaling cascade (Liao and Homcy 1993; Marceau, et al. 2002). In different other systems, B2R was also shown to activate multiple signaling pathways including G $\alpha_{i/o}$ (Ewald, et al. 1989) such as in rat dorsal root ganglion neurons or G $\alpha_{12/13}$ such as in fibroblast cell lines, however, very little is known about their cellular functions (Gohla, et al. 1999). The coupling of B2R to G $\alpha_{q/11}$ induces the activation of PLC, which in turn

activates DAG and IP₃, leading to an increase in the intracellular concentration of Ca⁺⁺. In synergy with Akt activation, the release of Ca²⁺ in the cytosolic milieu generates endothelial nitric oxide synthase (eNOS), mainly responsible for regulating the vascular tone as a vasodilator (Fig. 10). Moreover, B₂R signaling pathway also activates the arachidonic acid cascade, resulting in prostaglandins (PGI₂, PGE₂) production sharing similar vascular functions as NO (Leeb-Lundberg, et al. 2005). The activation of B₂R stimulates ERK1/2 signaling pathway via two different mechanisms, whether by the agonist-driven G $\alpha_{q/11}$ and G $\alpha_{i/o}$ activation or by transactivation of the EGFR, depending on the tissue/cellular system. However, moments after receptor activation, B₂R signaling is terminated as soon as the receptor undergoes desensitization and internalization. In contrast, B₁R is a constitutively internalized receptor, and only in presence of ligand it becomes resistant to desensitization and internalization and couples to G $\alpha_{q/11}$ and G $\alpha_{i/o}$ (Leeb-Lundberg et al. 2005; Marceau et al. 2002).

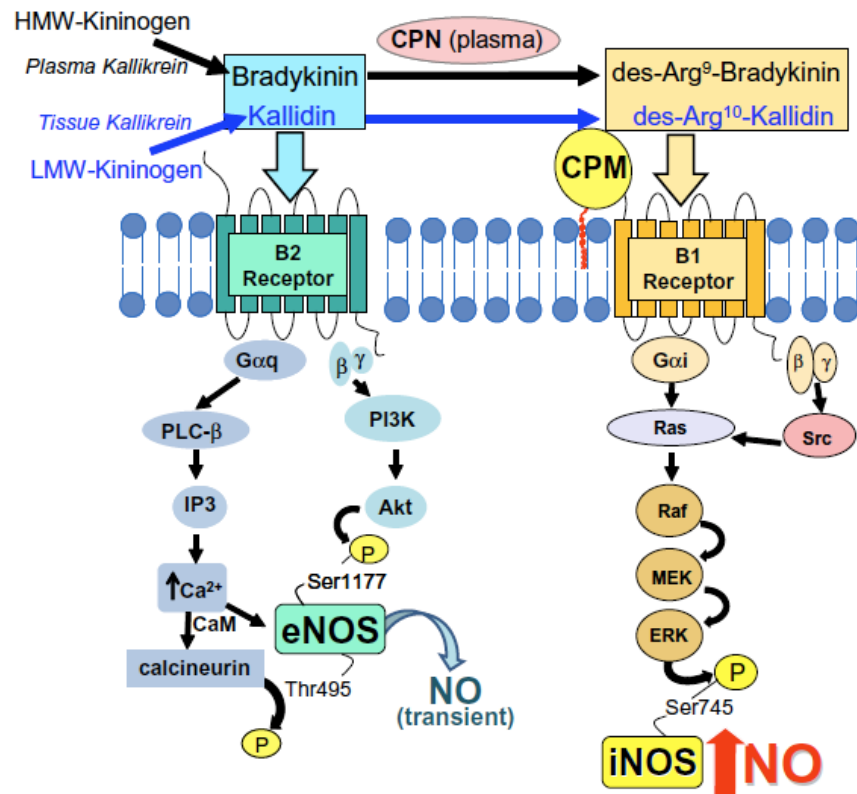


Figure 10. The bradykinin receptors signaling pathways.

Generated from plasma and tissue kallikrein, the high (HMW) and low (LMW) molecular weight kininogen are precursors of both bradykinin and kallidin, respectively. The membrane-bound carboxypeptidase M (CPM) or the soluble plasma carboxypeptidase N (CPN) converts the bradykinin and kallidin to des-Arg⁹-bradykinin and des-Arg¹⁰-kallidin through the removal of their C-terminal Arg. In endothelial cells, B2R signaling results in the activation of eNOS (nitric oxide synthase) by Akt-mediated phosphorylation of Ser1177 and calcineurin-mediated dephosphorylation of Thr495, leading to a transient NO production. On the other hand, B1R generates a prolonged NO response mainly due to the activation of iNOS (inducible isoform) via the Gα_i/Src-mediated MAPK signaling pathway (Kuhr et al. 2010).

3.2. The pathophysiological role of bradykinin receptors

Bradykinin receptors play important roles in various pathological conditions, such as cardiovascular diseases, inflammation and cancer. Both B1 and B2 receptors are expressed in cardiomyocytes and cardiovascular tissue, and once activated, these receptors serve as hypotensive factors, causing a decrease in the heart rate and blood pressure (Gouin, et al. 1996; Ribuo, et al. 1993). The expression of bradykinin receptors is upregulated under pathological conditions, such as in the case of myocardial infarction (Tschope, et al. 2000). Clinical studies showed that the survival rate of post-myocardial infarct patients was associated with the increase of the KKS, including kallikrein, kininogen and BK (Hashimoto, et al. 1978). Thus, the activation of B2R during pathological conditions was likely suggested to promote cardioprotective effects. Indeed, several studies using myocardial infarction or heart failure models have reported several cardioprotective effects of B2R mainly caused by NO production in endothelial cells (Groves, et al. 1995), inhibition of cell proliferation in fibroblasts (McAllister, et al. 1993) and a decrease of left ventricular hypertrophy (Wollert, et al. 1997). Another example of this is a study showing that the administration of exogenous kinins (BK and des-Arg⁹-BK) in the isolated heart of an ischemia rat model decreased the damaged area upon myocardial infarction and such effects were completely abolished when using HOE140, a known antagonist of the B2R (Lagneux, et al. 2003). Because of their vasodilatation properties, Kinins also participate in treating hypertensive patients. The angiotensin-converting enzyme (ACE), which catalyses the conversion of angiotensin I to angiotensin II (the active form) is primary involved in the renin-angiotensin system. This enzyme, which increases blood pressure by promoting vasoconstriction, is also known to

degrade kinins. Thus, in order to treat hypertension, ACE inhibitors are used for a better control of blood pressure, and other than blocking the angiotensin synthesis cascade, several studies showed that beneficial effects were also due to an increase of kinins in plasma (Marceau and Regoli 2004).

On the other hand, bradykinin receptors are involved in various inflammatory responses, such as in the case of pain, heat, redness and swelling. Pro-inflammatory effects observed in pancreatitis, arthritis and asthma were partly due to the increase of local concentrations of BK. The activation of B1R and B2R signaling pathways is known to induce the release of cytokines, generally associated with the activation of nuclear factor-kappa B (NF- κ B), and promote the release of several other substances involved in acute and chronic inflammatory processes (Chen, et al. 2004a; Marceau, et al. 1983; Merino, et al. 2005).

Other evidence showed that KKS is also involved in cancer. In fact, the pro-inflammatory properties of kinins, which promote vasodilatation and vascular permeability play a pivotal role in cancer metastasis. *In vitro* and *in vivo* studies using tissue specific inhibitors of kallikrein showed a slower progression of breast cancer mainly due to the diminished invasiveness of cancer cells (Wolf, et al. 2001). Another study on mice bearing sarcoma cells showed that the administration of B2R antagonist, HOE140, significantly decreased angiogenesis and diminished tumor weight (Ishihara, et al. 2001). In contrast, B2R agonist, RMP-7, was selected for clinical trials because of its high potency of inducing permeability of the blood-brain barrier which significantly increased the uptake of the chemotherapeutic agents in rat glioma models (Emerich, et al. 1998; Shimuta, et al. 1999). Because of the various properties associated to bradykinin

receptors, it has been a great challenge for drug discovery to assign safe therapeutic targets while balancing between the cardioprotective, pro-inflammatory and cancer-related consequences.

4. The vasopressinergic system

Vasopressin (AVP) is a neurohypophysial nonapeptide (Cys¹-Tyr²-Phe³-Gln⁴-Asn⁵-Cys⁶-Pro⁷-Arg⁸-Gly⁹) synthesized in the hypothalamus and released from the posterior pituitary gland as neurosecretory vesicles. It is mainly responsible for regulating the osmotic and volume homeostasis of body fluids as well as involved in several central functions such as learning and memory (Caldwell, et al. 2008; Donaldson and Young 2008). In terms of osmoregulation, the vasopressinergic system is highly sensitive, such as less than 1-2% of increase in plasma osmolarity can be controlled only by releasing very small amounts of vasopressin (Mutlu and Factor 2004). Besides physiological conditions, vasopressin circulating levels can also be increased in several pathological states, such as hypoxia and acidosis (Holmes, et al. 2001).

Vasopressin cellular responses are mediated by three GPCRs; vasopressin V1 (or V1a), V1b (referred to as V3) and V2 receptors. V1R is expressed in vascular smooth muscle cells acting as vasoconstrictor, V3R is found in the anterior pituitary involved in the adrenocorticotrophic hormone secretion, and V2R is mainly found in the renal collecting duct, specifically located on the basolateral membrane of epithelial cells and mainly responsible for water reabsorption (Bankir 2001; Maybauer, et al. 2008). V1R and V3R are known to couple to G $\alpha_{q/11}$, activate PLC, and increase the intracellular levels of Ca²⁺, which will induce vasoconstriction. However, V2R activates the AC through G α_s

coupling and promote cAMP accumulation, leading to PKA activation. The catalytic subunits of PKA bind aquaporin-2-containing endocytic vesicles, which in turn fuse into the luminal membrane of epithelial cells and increase the membrane permeability. Aquaporine-2 (AQ-2) is a water channel that play pivotal role in determining the ability of the collecting duct to reabsorb water in order to maintain an equilibrated osmolarity. It is generally stored in endocytic vesicles at basal state, and undergo exocytosis upon AVP-mediated stimulation. A detailed mechanism of the V2R and AQ-2 mechanism of action is illustrated in Fig 11 (Bichet 2009).

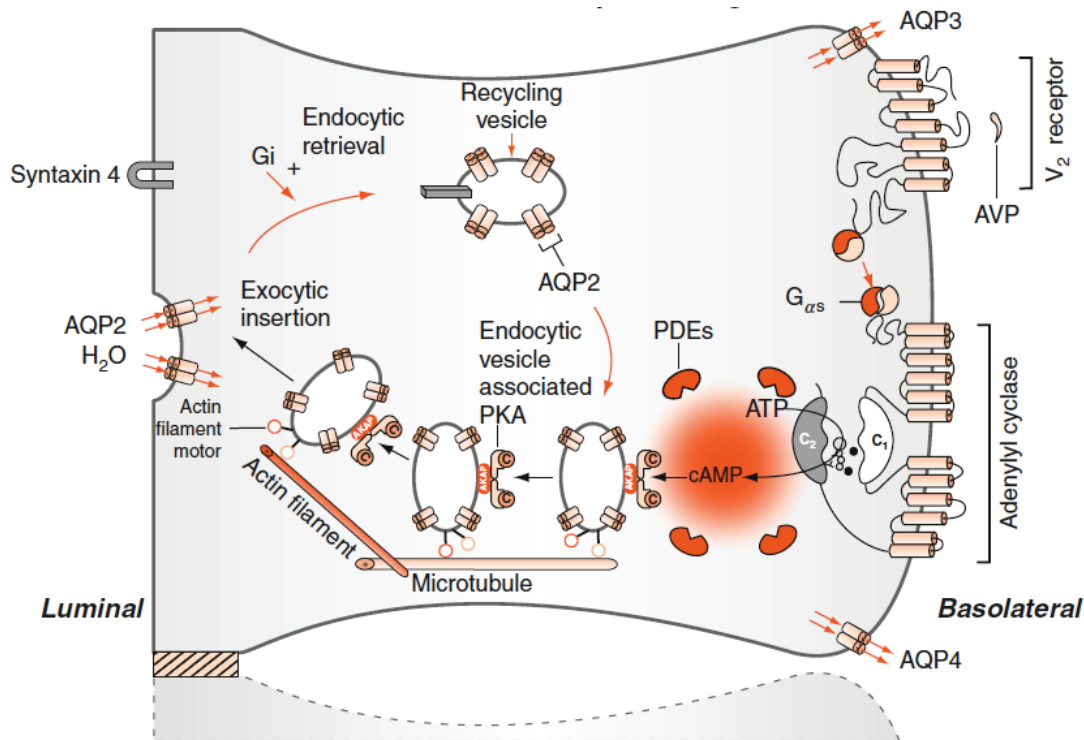


Figure 11. The vasopressin V2 receptor signaling effect on renal water reabsorption.

At the basolateral membrane, vasopressin (AVP) binds vasopressin V2 receptor (V2R), promotes G_{α_s} signaling pathway and activates adenylyl cyclase (AC) which converts ATP to cAMP. The cAMP interacts with protein kinase A (PKA) and induces conformational rearrangements that leads to the dissociation of its catalytic subunits (C). The activated subunits then bind aquaporin-2 (AQP2) mainly located in endocytic vesicles. Water channels fuse to the luminal membrane via exocytic insertion, thus increasing the water permeability of the collecting medullary duct. Vesicles movement to the plasma membrane is mediated by microtubules and actin filaments. In the absence of AVP, AQP2 undergo endocytic retrieval and return back to recycling vesicles, thus diminishing the water reabsorption rate. AQP3 and AQP4 are constitutively activated water channels located at the basolateral membrane (Bichet 2009).

The binding of AVP to V2R promotes receptor desensitization through phosphorylation and recruits β -arrestins for internalization (Bowen-Pidgeon, et al. 2001). Once receptor signaling is terminated, one would expect that the activation of AQ-2 would be altered. Indeed, AQ-2, which is resistant to internalization during V2R activation, was shown to recycle back from the plasma membrane and accumulates in 'recycling' vesicles when AVP was removed, resulting in an impaired membrane permeability (Bouley, et al. 2006). Moreover, V2R signaling pathways were also shown to be involved in several other processes such as sodium reabsorption via the activation of epithelial Na^+ channel (ENaC) as well as urea reabsorption by activating urea transporters (UTA1) in the distal nephron (Fenton 2009; Sands and Layton 2009).

In terms of clinical implications, the congenital Nephrogenic Diabetes Insipidus (NDI) is an example of a kidney disease directly associated to the AVP-V2R-AQ-2 system and generally caused by several mutations of the V2R (Bichet 2009). Hence, the process of V2R signaling and trafficking and its effect on AQ-2 has been intensively investigated over the past years and V2R is now considered as a potential target for treating multiple kidney diseases.

5. Beta-arrestins

Arrestins are divided into four homologous members that belongs to two subfamilies: visual or sensory arrestins composed of arrestin 1 (previously referred as *S-antigen* or 48 kDa protien) and arrestin 4 (also called X-arrestin or cone arrestin) and non-visual arrestins including β -arrestin-1 (also known as arrestin-2) and β -arrestin-2 (also known as arrestin-3) (Gurevich and Gurevich 2006). Discovered in 1970s, the

arrestin protein, referred to as *S-antigen* (for soluble antigen), was first described as an immunogenic retinal antigen implicated in an autoimmune disease causing eye inflammation (Wacker, et al. 1977). Meanwhile, Hermann Kuhn's work described a light-dependent binding of a 48 KDa protein to rhodopsin (Kuhn 1978; Wilden, et al. 1986). Upon light activation, this retinal protein was not only shown to bind phosphorylated rhodopsin but also blocks its G protein signaling through the inhibition of the cGMP phosphodiesterase activity (Miller, et al. 1986; Zuckerman and Cheasty 1986). Based on its 'arresting' function, the 48 kDa protein was then named arrestin. Subsequently, more evidence has shown that arrestins are directly linked to receptor endocytosis. Studies have demonstrated that knocking down β -arrestins diminishes the internalization of β 2AR (Mundell, et al. 1999). Beta-arrestins were then shown to bind several components of the endocytic machinery such as clathrin (Goodman, et al. 1996) and AP-2 (Laporte, et al. 1999). This endocytic function was further characterized and it is now well established that arrestins are not only involved in receptor trafficking, but also in endosomal signaling and moreover, promote various biological responses which made the arrestin a potential therapeutic target for many pathological diseases.

5.1. Beta-arrestin structure and mechanism of action

Over the past years, crystal structures of all arrestins have been revealed, starting with the visual arrestin that has been extracted from bovine rod outer segments (Granzin, et al. 1998), followed by β -arrestin-1 (Milano, et al. 2002), arrestin 4 (Sutton, et al. 2005), and finally β -arrestin-2 (Zhan, et al. 2011). The homologous structure, which is well conserved between the arrestin members, comprises two concave lobes of anti-

parallel β -sheets (C- and N- domains) connected by the hinge domain and the polar core, and an α -helix located in the back of the N-terminal region (Fig. 12) (Vishnivetskiy, S. A. et al. 2002).

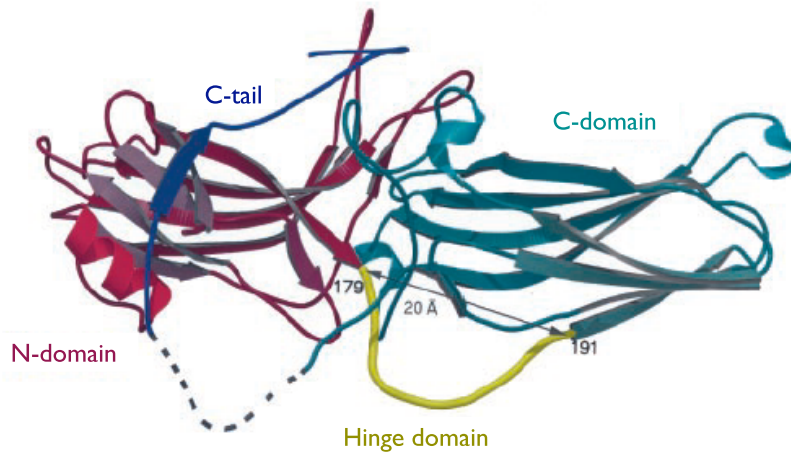


Figure 12. Schematic representation of the crystal structure of arrestin.

The N-domain lobe is in red, the C-domain in dark green and the C-tail in blue. The hinge domain is in yellow, denoted from residue 179 to 191 and limited by a distance of 20 Å. The dashed line in blue represents a disordered region of the arrestin (adapted from (Vishnivetskiy, et al. 2002)).

The structural features of arrestins provided important clues on the mechanism underlying their agonist-dependent binding to GPCRs. Therefore, it is now well known that arrestins recruitment to activated receptors requires two distinct types of sensors: a ‘phosphorylation sensor’ mediated by the C-terminus and/or the ICL3 of the receptor which is generally phosphorylated by GRKs and an ‘activation sensor’ which recognizes the active form of the receptor, mainly detected by the conformational changes of the core (Gurevich and Gurevich 2006). The arrestin/receptor complex interaction was investigated by mutagenesis experiments and showed that the affinity of the arrestin to rhodopsin was clearly increased when the receptor was phosphorylated upon light-activation (Gurevich and Benovic 1993). Other *in vitro* studies using binding assays also showed that the phosphorylation of the β 2AR increased the affinity of β -arrestin-1 from approximately 60 nM to 1.8 nM (Sohlemann, et al. 1995). Recently, a new study revealing the crystal structure of the activated β -arrestin-1 bound to a GPCR phosphopeptide showed that the activation of the arrestin exhibit conformational changes when compared to the inactive form (Shukla, et al. 2013). These observations are supported by previous studies showing that visual arrestin also undergo conformational rearrangements upon activation, and this process induces a high affinity binding state with phosphorylated rhodopsin (Gurevich and Gurevich 2004).

Mechanistically, the binding of arrestin to the receptor requires a two step process of intramolecular interactions: the polar core interaction and the ‘three-element’ interaction. The polar core, a common region for all the arrestins, stabilizes the inactive conformation of the arrestin by forming a network of five electrostatic residues (D26, R169, D290, D297 and R393 for β -arrestin-1 or D30, R175, D296, D303 and R382 for

visual arrestin) (Fig. 13) (Gurevich, et al. 2014). In other words, at basal state, the N- and C-terminal domains of arrestin, which contains receptor-binding motifs in their concave surface, are locked in an open/elongated structure. This steady conformation was shown to be mainly maintained by the Arg 175 (or Arg 169 in β -arrestin), which forms a salt bridge with Asp 296. This key residue serves as a phosphorylation sensor, which binds the negatively charged phosphate residues associated to the phosphorylated receptor. Disruption of the salt bridge by performing mutations of either Arg175 to Glu175 or Asp296 to Arg296 resulted in a phosphorylation-independent binding of visual arrestin and β -arrestin-1 to the activated receptor (Gurevich and Benovic 1997; Kooor, et al. 1999; Vishnivetskiy, et al. 1999).

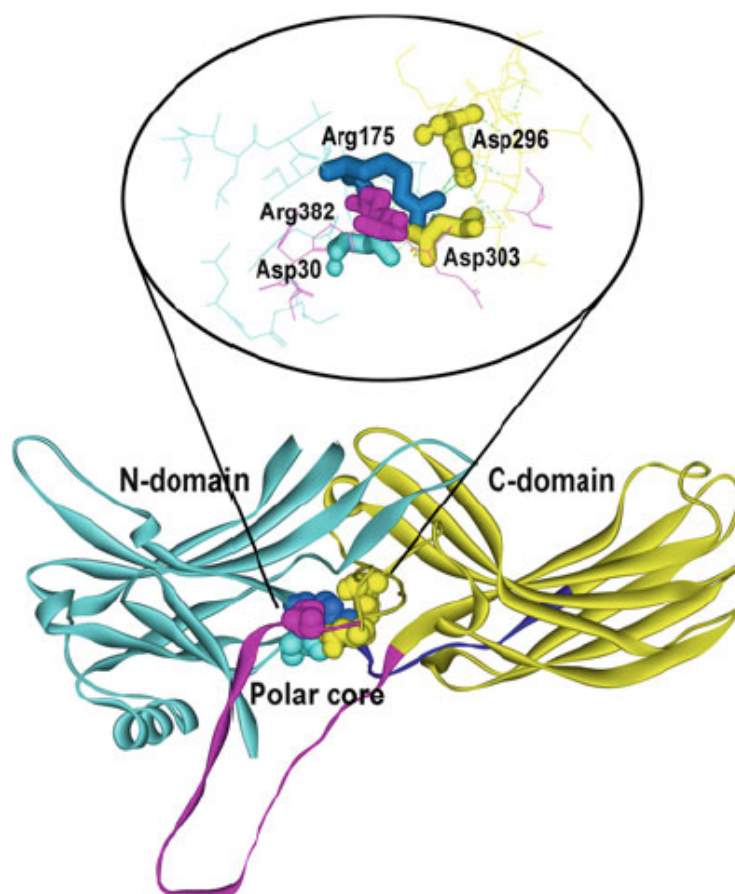


Figure 13. The polar core of the arrestin.

The polar core is a key phosphate sensor that stabilizes the arrestin in a basal conformation due to five important residues forming a network of several ionic interactions: three negatively charged residues; Asp30 (light blue), Asp 296 (yellow) and Asp303 (yellow); two positively charged residues; Arg175 (dark blue) and Arg382 (magenta). N-domain (light blue), C-domain (yellow), hinge domain (dark blue), and C-tail (magenta) (Gurevich et al. 2014).

The second set of interactions involves the ‘three-element’ interaction which occurs between the β -strand I, α -helix I and the extended C-terminal domain that stabilizes the polar core and locks the arrestin in an inactive conformation (Fig. 14) (Gurevich et al. 2014; Hirsch, et al. 1999; Shukla et al. 2013).

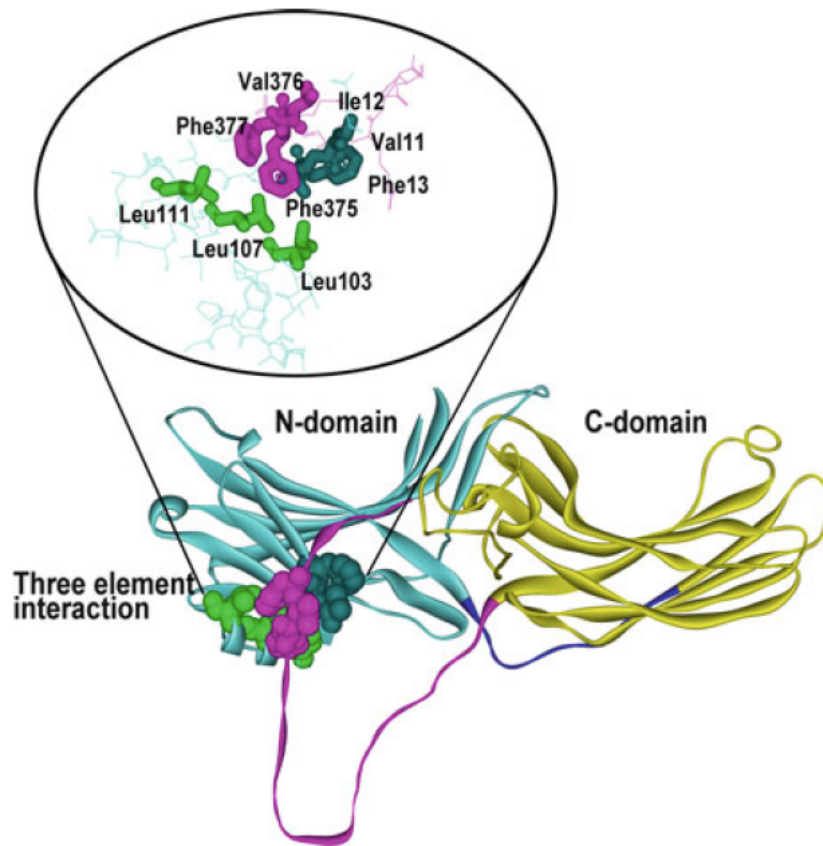


Figure 14. The three-element interaction of the arrestin.

The three-element interaction, which stabilizes the arrestin in a basal conformation, consists on several interactions between the hydrophobic residues in the β -strand XX of the C-tail (Phe375, Val376, Phe377, magenta) with residues of the β -strand I (Val11, Ile12, Phe13, dark blue) and the α -helix I (Leu103, Leu107, Leu111, green) located at the N-domain. N-domain (light blue), C-domain (yellow), hinge domain (dark blue), and C-tail (magenta) (Gurevich et al. 2014).

Upon agonist-induced activation of GPCRs, the phosphorylated residues of the receptor from the carboxyl tail will bind the arrestin and destabilize the intramolecular interactions from the polar core and the 'three-element' region (Shukla et al. 2013). This action will induce the closing of the N- and C- terminal region onto the receptor, in a '*clamshell*'-like conformation and triggers the release of the arrestin carboxyl tail (Gurevich and Gurevich 2004). In addition, other than the interdomain rearrangements, multiple movements in the arrestin loops were also detected upon activation. Notably, conformational changes were observed in the finger loop (residues 63-75), the middle loop (residues 129-140) and the lariat loop (residues 274-300) and mutational analysis have shown that such movements are in part responsible for the activation of the arrestin and its binding to the receptor (Fig. 15A) (Shukla et al. 2013). Moreover, the hinge loop (position 179-191 for visual arrestin or 173-185 for β -arrestin) that connects the N- and C- extremities of the arrestin has also been shown to be necessary for maintaining receptor/arrestin complexes in a stable conformation (Fig. 12). Sequential deletion in the hinge domain of visual arrestin showed a decrease in its ability to bind light-phosphorylated rhodopsin (Vishnivetskiy et al. 2002). On the other hand, structural observations also suggested that the N- and C- terminal domains of the activated arrestin undergo a 20° rotation from the central axis which, facilitates its interaction with the receptor (Fig 15B) (Shukla et al. 2013). These conformational rearrangements suggest that arrestin binds the receptor only from the concave domains, which leaves the convex motifs exposed to various binding partners, such as clathrin, AP-2, phosphoinositides, and other signaling effectors important for regulating the trafficking and downstream signaling of GPCRs.

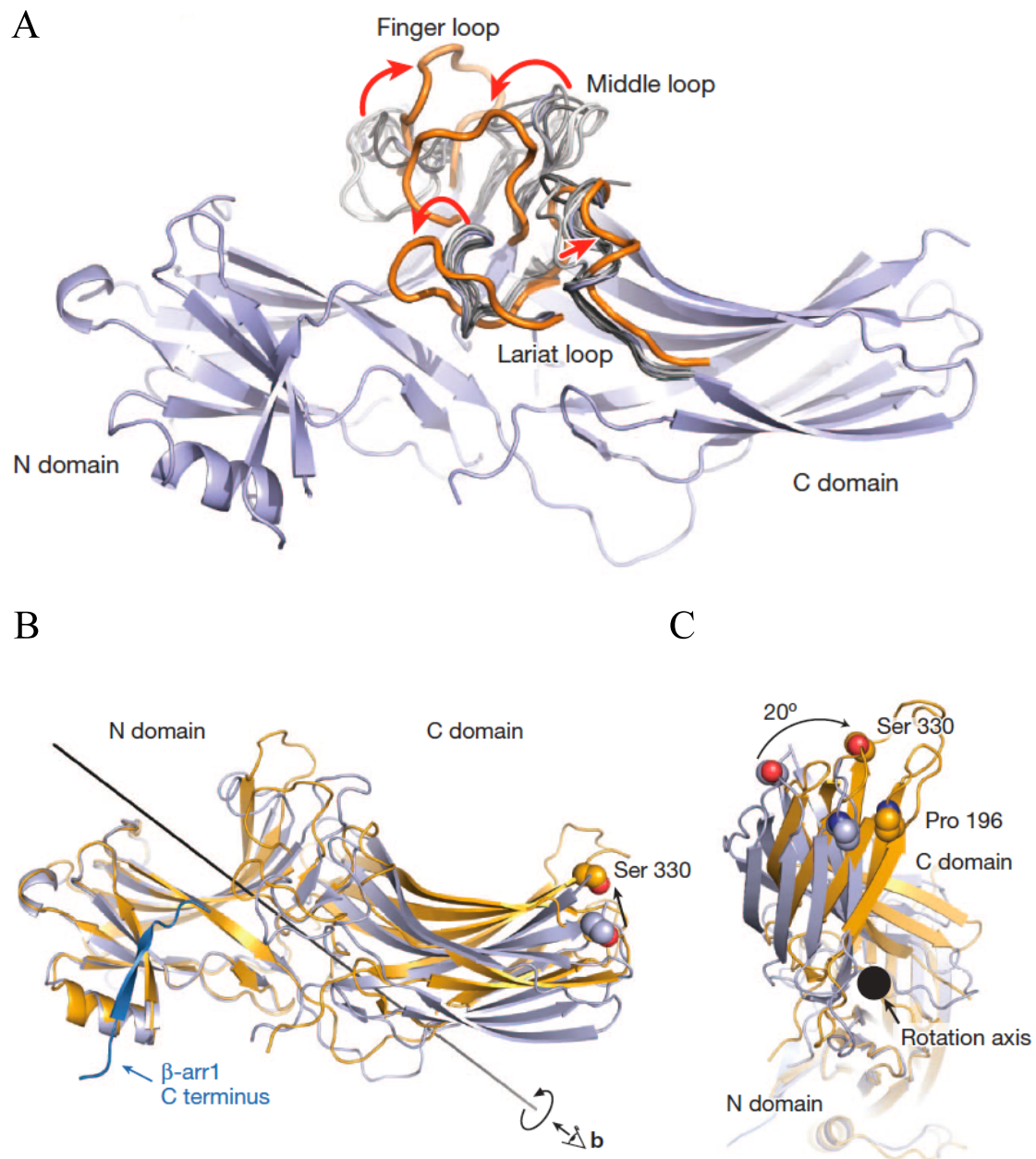


Figure 15. Schematic diagram of the conformational changes associated to the active form of β -arrestin-1.

(A) Illustration of the three dimensional structure of β -arrestin-1 using a cartoon visualization of the α -helices and the β -sheets, showing the structural features of the

finger, middle and lariat loops of the inactive (grey) form of arrestin and the conformational rearrangements associated to its active form (orange). (B) Representation of the 20° rotational movement of the arrestin when switching from the inactive (light blue) to the active (gold) form. The release of the C-terminus tail is showed in blue; the rotation axis is illustrated by a black line and Ser330 residue is an example of an amino acid rotational movement. (C) View of the arrestin movement from the C-terminus domain (Shukla et al. 2013).

5.2. Role of β -arrestins in signaling

In the classical model of 7 TMs receptor activation, signaling was only mediated by G protein coupling, whereas, arrestins were responsible for receptor desensitization. Without any enzymatic activity, the arrestins were known to only bind GRK-phosphorylated receptors, uncouple G protein signaling and direct the receptors into endosomal compartments through the internalization machinery. However, the current model suggests that GPCR signaling is not only mediated by G protein, but arrestins are also involved in a plethora of signaling pathways which will be covered in this following section (Rajagopal et al. 2010).

5.2.1. Discovery of arrestin signaling

By the end of 1990s, several studies using either dominant negative forms of dynamin and/or β -arrestin-1 or pharmacological inhibitors of clathrin-mediated internalization suggested that receptor internalization is required for a full activation of the GPCR-induced extracellular signal-regulated kinases (ERK1/2) signaling (Lin, et al. 1998; Luttrell, et al. 1997). Then, the first evidence of β -arrestin-mediated signaling was

reported in a study showing that β -arrestin-1 recruits activated c-Src, a non-receptor tyrosine kinase, and this protein-protein interaction was necessary for β 2AR-mediated MAPK activation (Luttrell, et al. 1999). Subsequently, another study on the neurokinin receptor (NK1) also showed that the recruitment of Src to β -arrestin-1 was crucial in maintaining the mitogenic and anti-proliferative effects of agonist-stimulated receptor (DeFea, et al. 2000). Another study on the AT1R also demonstrated the important role of β -arrestins in cellular signaling (McDonald, et al. 2000). Over-expressing either β -arrestin-1 or β -arrestin-2 increased the uncoupling of the $G\alpha_q$ protein from the receptor, which blocked the inositol phosphate accumulation, mainly mediated by PLC β . However, an increase in the AT1R-mediated ERK1/2 activation was only observed in β -arrestin1/2 over-expressed conditions. Therefore, results suggest that arrestins are involved in the MAPK signaling pathway, thus promoting the transition of the receptor from a signaling state to another (Tohgo, et al. 2002).

5.2.2. The relationship between arrestin and ERK1/2 signaling

5.2.2.1. ERK1/2 signaling pathway

MAPK are a family of serine threonine kinases implicated in a wide range of cellular responses, including proliferation, mitosis, differentiation and apoptosis. The ERK1/2 signaling pathway is activated by almost all GPCRs, cytokines and receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor (EGFR) and insulin growth factor receptor (IGFR) (Boulton, et al. 1990; Raman, et al. 2007). MAPK signaling cascade consists of a series of kinases, where either b-Raf or c-Raf (MAPKKKs) phosphorylates and activates MEK1 and MEK2

(threonine/tyrosine MAPKKs), which then stimulate ERK1/2 via phosphorylation (Cargnello and Roux 2012). In the inactive form, ERK1/2 kinases are primarily located in the cytosol, but upon activation, they translocate into the nucleus (Chen, et al. 1996). Activated ERK1/2 phosphorylates a variety of substrates, some of them associated to the plasma membrane, such as calnexin, others in the cytoplasm, such as death-associated protein kinase (DAPK) and p90 ribosomal S6 kinases (RSK) but most of them are transcription factors from the nucleus, including *c-Fos*, *c-Myc* and *Elk-1* (Reviewed from Yoon, S. and Seger, R. 2006).

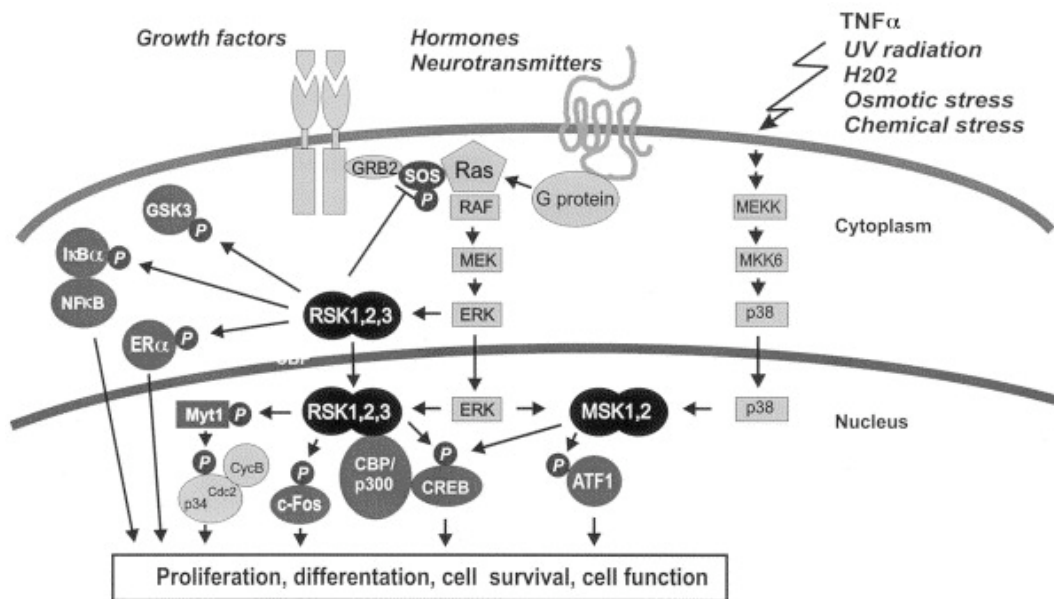


Figure 16. The ERK1/2 signaling pathway.

The specificity and efficiency of MAPK signaling pathway are mostly regulated by the binding of its successive kinases to scaffolding proteins (Burack and Shaw 2000). The interaction of MAP kinases to their corresponding substrates consists of two distinct motifs: The first one is an ERK1/2 binding site, called D domain, which is a conserved sequence composed of a series of basic residues followed by hydrophobic amino acids; Lys/Arg-Lys/Arg-X₂₋₆- ϕ -X- ϕ , where ϕ stands for a hydrophobic residue, such as Iso, Leu or Val (Tanoue and Nishida 2003). The second one is an ERK-phosphoacceptor site, composed of PES/TP and generally found in most ERK1/2 substrates (Pearson and Kemp 1991; Songyang, et al. 1996). While the mechanism involving the MAPK signaling pathway is well defined, the effect of ERK1/2 on GPCRs/endosomal scaffolds remains poorly characterized.

5.2.2.2. Beta-arrestin-mediated ERK1/2 signaling pathway

During the process of internalization, β -arrestin scaffolds with multiple components of the MAPK signaling pathway. Co-immunoprecipitation studies first showed that c-Raf1, MEK1 and ERK2 are associated with β -arrestin2, and the binding of MEK1 and ERK2 to the arrestin was increased when c-Raf1 was co-expressed in the cells. Receptor/Arrestin/c-Raf1/MEK1/ERK2 complex assembly was regulated in an agonist-dependent manner (Luttrell, et al. 2001). Similar observations were also described on ligand-induced protease-activated receptor 2 (PAR2) where β -arrestin-1 was found in complex with the receptor, c-Raf1 and phosphorylated ERK2 (DeFea et al. 2000). In addition, the Laporte laboratory has shown that such signaling effectors can also regulate the trafficking of β -arrestins-bound GPCRs (Fessart, et al. 2005; Fessart, et

al. 2007; Lin, et al. 1997). For instance, phosphorylation of β -arrestin-1 by ERK1/2 in the C-terminal domain modulates its ability to bind clathrin and AP-2, and impedes β 2AR internalization (Lin, et al. 1999). The recruitment of Src kinase to receptor/ β -arrestin-2 complexes induces the phosphorylation of β -subunit of AP-2 and dynamin, which facilitates angiotensin II type 1 (AT1R) and the β 2AR internalization (Ahn, et al. 1999; Fessart et al. 2007).

When the idea of β -arrestin as a MAPK signaling scaffold was established, research studies have focused on the biological consequences associated to the arrestin signaling function. Since most GPCRs were known to induce ERK1/2 activation, the downstream outcome of this signaling pathway was already identified. For example, the heterotrimeric $G\alpha_q$ was shown to induce the activation of PKC through PLC β , which in turn activates c-Raf1 leading to the direct phosphorylation of ERK1/2 (Kolch, et al. 1993). Subsequently, ERK1/2 translocates to the nucleus, phosphorylates and activates the Ets domain-containing transcription factor Elk-1, which in turn promotes the expression of immediate-early genes, mostly involved in cell cycle progression. The $G\alpha$ -dependent activation of ERK1/2 consists of a rapid signal onset (2-5 minutes), followed by a progressive decrease of the signaling intensity caused by receptor desensitization via GRK-mediated phosphorylation. However, studies have shown that the binding of β -arrestin to the receptor switches the GPCR signaling into a distinct temporal and spatial ERK1/2 signaling mode. Beta-arrestin-dependent signaling is characterized by a slower onset of signal intensity, which remains for 30 to 60 minutes of ERK1/2 activation (Fig. 17) (DeWire, et al. 2007).

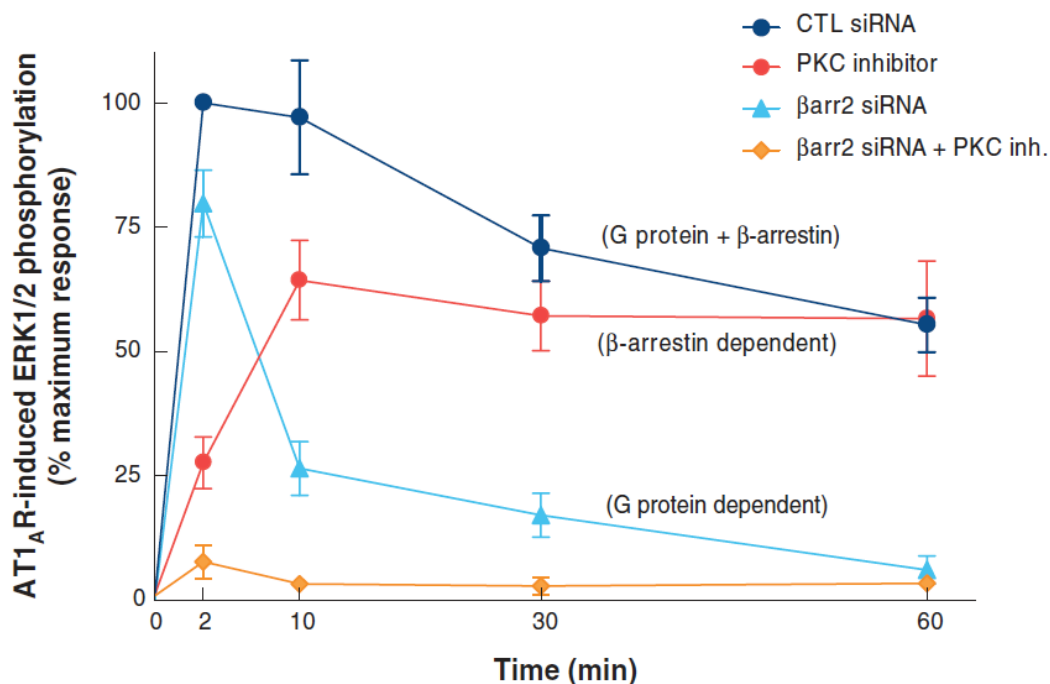


Figure 17. β -arrestin versus G protein signaling in AT1R-induced ERK1/2 phosphorylation.

Cells were either treated with PKC inhibitor or transfected with β -arrestin-2 siRNA before being challenged with AngII (100 nM) for the indicated time. Stimulation curves show that G protein signaling occurs at early time points (2 min) whereas β -arrestin signaling is found at later time points (30 and 60 min) of AngII stimulation (DeWire et al. 2007).

Such signaling behaviour was demonstrated on several GPCRs, including AT1R (Ahn, et al. 2004) and β 2AR (Shenoy, et al. 2006). Besides the late-phase activation of ERK1/2, β -arrestin-dependent signaling also affects kinases sub-cellular localization. For instance, a study using the AT1R showed that the over-expression of β -arrestin-1 and β -arrestin-2 inhibited the G protein signaling (*e.g.* phosphoinositide hydrolysis) but promoted a sustained ERK1/2 activity, which was suggested to be an arrestin-dependent effect. However, ERK-dependent transcription (*e.g.* Elk-1 transcription factor) was diminished in such conditions (Tohgo et al. 2002). Results from confocal microscopy also showed that β -arrestins prevent ERK1/2 from nuclear translocation leading to its accumulation in early endosomes with the receptor (Luttrell et al. 2001). As a consequence, multiple biological responses have been associated to β -arrestin-dependent signaling, such as cytoskeletal rearrangement and chemotaxis (Barnes, et al. 2005; Hunton, et al. 2005).

Over the years, the arrestin signaling concept has evolved and when this phenomenon was applied in terms of biased signaling, where specific ligands direct receptor signaling through either G protein or β -arrestin pathways, the arrestin was then qualified as a potential therapeutic target for several pathological conditions. One of the best cited example is the [Sar⁽¹⁾, Ile⁽⁴⁾, Ile⁽⁸⁾]-angiotensin II (SII), an agonist of AT1R, known to selectively induce β -arrestin-2/ERK1/2 signaling pathway and to promote cell migration, protein synthesis and proliferation during *in vitro* assays and increased cardiac performances in ischemic rat model (Ahn, et al. 2009; Aplin, et al. 2007; Hostrup, et al. 2012; Rajagopal, et al. 2006).

5.2.2.3. Beta-arrestin/receptor endosomal signaling

One of the key control of β -arrestin-dependent signaling actions is the stability of the complex that joins the arrestin to the receptor in the endosomes, called ‘signalsome complex’. Whether β -arrestins complex with receptors into endosomes or whether they dissociate from them at the plasma membrane during endocytosis, have been used as criteria to regroup GPCRs into different classes (Oakley, et al. 2000). For instance, Class A receptors form short-lived complexes with both β -arrestins at the plasma membrane and are rapidly recycled to the cell surface. For example, the β 2AR and lysophosphatidic receptor (LPA) form transient interactions with β -arrestins, which dissociates from the receptors right after endocytosis. This transitory receptor/ β -arrestin complex state did not show sustained ERK1/2 endosomal activity, but instead generated a transcriptionally active ERK1/2 population, able to translocate into the nucleus and induce cell proliferation (Shenoy et al. 2006). On the other hand, Class B receptors form longer-lived complexes with β -arrestins into endosomes, and are generally retained inside the cell even after the removal of the ligand from the extracellular milieu (Fig. 18). An example of this class is the AT1R, which internalizes with β -arrestins and promotes arrestin-mediated signaling from the endosomes. Based on the high affinity binding state with β -arrestins, class B receptors are more plausible to undergo degradation via the lysosomal process. We have recently showed a hybrid trafficking behaviour of β -arrestin with the B2R (Khoury, et al. 2014b). Similarly to class B receptors, B2R internalizes with β -arrestins into endosomes upon agonist stimulation (Simaan, et al. 2005). However, these complexes are short-lived in endosomes, and receptors rapidly recycle back to the plasma membrane following agonist removal.

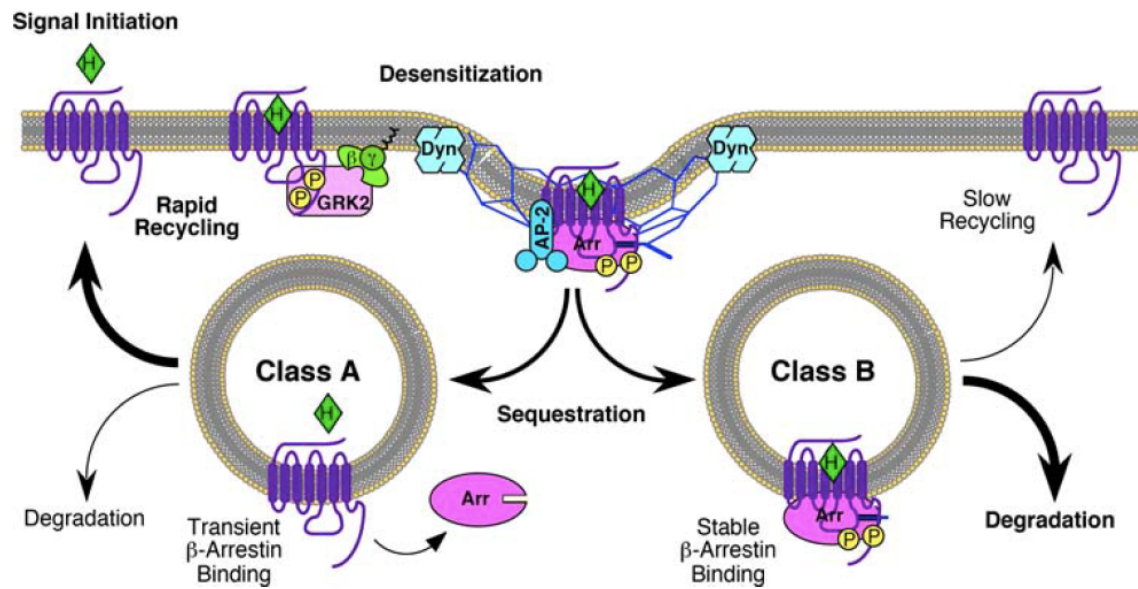


Figure 18. The arrestin/GPCR complex interactions.

The complex stability between the arrestin and the receptor in the endosomes forms two classes of GPCRs: Class A GPCRs, which are characterized by a transient β -arrestin binding and favors receptor rapid recycling over degradation; Class B GPCRs, which forms a stable interaction with β -arrestin and are either targeted to lysosomes for degradation or undergo slow recycling (Kendall and Luttrell 2009).

5.2.3. Beta-arrestin diverse signaling pathways

5.2.3.1. p38 MAPK signaling pathway

p38 MAPK consists of four different isoforms, α , β , γ and δ , and promotes gene transcriptional activation in response to inflammatory cytokines and growth factors (DeWire et al. 2007). One of the first examples of arrestin-mediated activation of p38 was shown with the cytokine receptor US28, a GPCR encoded by the human cytomegalovirus (Miller, et al. 2003). The US28 is a 7TMs receptor constitutively phosphorylated by GRKs and recruits β -arrestin without agonist stimulation. A study performing mutations in the C-tail of US28 showed that the dissociation of the arrestin from the receptor inhibited p38 kinase activity. Parallel studies also showed that CXCR4 requires receptor phosphorylation and β -arrestin2 recruitment in order to enhance p38 activation (Sun, et al. 2002). The mechanism of the arrestin-mediated activation of p38 is not well characterized, however studies using biochemical approaches showed that apoptosis signal-regulating kinase 1 (ASK1) regulates p38 phosphorylation. Results suggested that receptor/arrestin complexes recruit several signaling effectors involved in MAPK activation, such as ASK1, and this MAPKKK will further activate MEK 3/6 and then phosphorylates p38 (Burack and Shaw 2000; DeWire et al. 2007). Cellular and physiological responses were also shown to be mediated by the arrestin-dependent p38 signaling cascade. For example, p38 activation via arrestin induces apoptosis in mouse embryonic fibroblasts (Yang, et al. 2012), regulates endothelin-mediated cell migration of mouse aortic smooth muscle cells (Morris, et al. 2012) and promotes hypertrophy and proliferation of astrocytes in the spinal cord and the brain (Bruchas, et al. 2006; Xu, et al. 2007).

5.2.3.2. c-Jun N-terminal kinase (JNK) signaling pathway

The third member of the MAPK cascade, JNK, was also shown to be activated by β -arrestin. The JNK family is encoded by three different genes, JNK 1, -2 and -3 with distinct properties. JNK1 and -2 are ubiquitously expressed, however, JNK3 is more abundant in the brain, the heart and testis (Mohit, et al. 1995). Using a yeast two-hybrid screen, JNK3 was first found in complex with β -arrestin-2, and subsequent studies showed that upstream kinases, such as MKK4 and ASK1, were also identified as arrestin binding partners responsible for the activation of JNK3 (Zang et al. 2009; Zhan et al. 2013). Colocalization of the active form of JNK3 with β -arrestin-2 was observed in cytosolic vesicles upon AT1R activation (McDonald et al. 2000; Miller, et al. 2001). Moreover, structural studies showed that the C-domain of β -arrestin-2 was important in the activation of JNK3. Specifically, Val-343 was identified as the key residue responsible for JNK3 phosphorylation in addition to other important residues, such as Leu-278, Ser-280, His-350, Asp-351, His-352 and Ile-353. Mutagenesis of β -arrestin-2 targeting these critical residues impaired the phosphorylation of JNK3, without affecting the ability of β -arrestin to bind ASK1, MKK4 and JNK3 (Seo, et al. 2011). Despite the high homology between arrestins sequences, β -arrestin-1 and the two visual arrestins did not facilitate JNK3 activation as shown with β -arrestin-2 (Song, et al. 2009).

5.2.3.3. Phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway

Another known signaling pathway involved in the arrestin function is the PI3K. The PI3K activated by growth factors, cytokines and various hormones, catalyzes the

phosphorylation of PI(4,5)P₂ to phosphatidylinositol-3,4,5-triphosphate (PIP₃) located at the plasma membrane. Upon PI3K activation, PIP₃ serves as a second messenger that binds and activates Akt through its PH domain, which results in the translocation of the Akt at the plasma membrane. Activated Akt, also referred to as protein kinase B (PKB), is mainly involved in various anti-apoptotic responses such as cell growth, proliferation and survival and many evidence showed that β -arrestin is pivotal in regulating the Akt signaling pathway. For instance, a study using the insulin-like growth factor 1 (IGF-1) receptor showed that β -arrestin-1 was responsible for activating the PI3K signaling pathway, which in turn activated Akt and induced anti-apoptosis. Moreover, when using mouse embryo fibroblasts (MEFs) lacking both β -arrestins, the IGFR failed to stimulate PI3K, however, the effect was completely restored when β -arrestin-1 was transfected exogenously (Povsic, et al. 2003). Another study also showed that β -arrestin-2 inhibited pro-apoptotic protein Bcl-2-associated death promoter (BAD) through the activation of the PI3K/Akt signaling pathway in vascular smooth muscle cells (Ahn et al. 2009).

5.2.3.4. Wnt/ β -catenin signaling pathway

β -arrestins are also implicated in the signaling of non-GPCR pathways, such as Wnt/ β -catenin signaling cascade. The binding of Wnt ligand to the 7TMs Frizzled receptor and its co-receptor low-density lipoprotein-related protein (LRP5/6) activates adaptor protein Dishevelled (DVL), and promotes the stabilization of β -catenin, which translocate to the nucleus for transcriptional activation. β -arrestin-1 and β -arrestin-2 bind phosphorylated DVL and play major role in inhibiting the degradation of the β -catenin complex (Bryja, et al. 2007; Chen, et al. 2001).

5.2.3.5. EGFR transactivation pathway

GPCRs can also activate the classical MAPK signaling pathways through transactivation of multiple receptors tyrosine kinases (RTK), such as the EGFR, the PDGFR and the IGFR. Transactivation can be mediated through the activation of metalloproteases (MMPs) or via ligand-dependent mechanism, where the 7TMs receptor activates a pathway that releases the membrane-bound EGF ligand by proteolytic cleavage leading to the activation of EGFR (Prenzel, et al. 1999). In addition to these two mechanisms, β -arrestin was also shown to link GPCRs to RTK through transactivation and in most cases it occurred in a Src-dependent manner (Kim, et al. 2008; Maudsley, et al. 2000). β -arrestin-mediated transactivation demonstrated distinct effects on cellular processes and physiological responses. An example of this is a β -arrestin-mediated transactivation of EGFR via agonist-bound β 1AR that was shown to promote the activation of cardioprotective pathways in mice undergoing chronic sympathetic stimulation (Noma, et al. 2007).

5.3. Functional regulation of β -arrestins

Over the past years, β -arrestin signaling function has been well characterized, and proteomic analysis showed that arrestins serve as scaffolding proteins that interact with a large number of binding partners. A recent study identified 71 proteins that bind to β -arrestin-1 and 164 proteins interacting with β -arrestin-2. These binding partners were associated with signaling transduction, cellular organization, nucleic acid binding and many others (Xiao, et al. 2007). It was thus suggested that arrestin/receptor complex

affinity state might be regulated by these arrestins binding proteins. Indeed, ERK1/2, known to directly interact with β -arrestins, was shown to phosphorylate β -arrestin-1 on Ser-412, and as a consequence, reduced β -arrestin-1-mediated β 2AR internalization. When using a dominant negative MEK1 mutant, the inhibition of ERK1/2 activity diminished arrestin phosphorylation, thereby increasing its ability to bind clathrin, hence, inducing receptor internalization (Lin et al. 1999). Regulation of β -arrestin-2 phosphorylation is also mediated by casein kinase II on residue Thr-382. Functional analysis using mutants of this key residue impaired the binding of arrestin to clathrin, AP-2 and Src, thus affecting receptor internalization (Kim, et al. 2002). Another study also reported that visual arrestin phosphorylation on Ser-366 significantly inhibits its interaction with clathrin (Kiselev, et al. 2000). All this suggests that arrestins endocytic function on GPCRs is in part regulated by a phosphorylation/dephosphorylation mechanism.

Other studies also showed that the stability of β -arrestin/receptor endosomal complexes correlates with the arrestins ubiquitination status (Shenoy and Lefkowitz 2003; Shenoy, et al. 2009). Upon receptor activation, β -arrestin binds E3 ubiquitin ligases, such as mouse double minute 2 homolog (Mdm2), neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) and tumor necrosis factor receptor-associated factor 6 (TRAF6) for ubiquitination and then interacts with ubiquitin-specific protease (USP33) for de-ubiquitination. The balance between both processes controls the stability of β -arrestin/receptor complex. For instance, upon β 2AR internalization, β -arrestin-2 undergoes ubiquitination followed by rapid de-ubiquitination, and this phenomenon causes transient interaction between the arrestin and the receptor,

leading to a fast recycling of the receptor to the plasma membrane. However, in the case of V2R, β -arrestin-2 is not de-ubiquitinated, which results in a stable receptor/arrestin complex (Shenoy et al. 2009). Unlike V2R and β 2AR, ubiquitination of β -arrestin-2 on position 11 and 12 (K11, K12) was shown to be crucial for maintaining a stable complex with internalized AT1R (Shenoy and Lefkowitz 2005).

In addition to the phosphorylation and ubiquitination sites, studies have shown that arrestins also contain sites for post-translational modifications that regulate endosomal complex stability. For example, nitrosylation of β -arrestin-2 on Cys 409 increases its binding affinity to clathrin and enhances β 2AR internalization (Ozawa, et al. 2008). SUMOylation (Small ubiquitin-like modifier modification) of β -arrestin-2 on Lys 400 also increases the ability of arrestin to bind AP-2 and promotes receptor internalization (Wyatt, et al. 2011).

Once in endosomes, receptors dissociate from the arrestin and recycle back to the plasma membrane and this phenomenon stands on the ability of the arrestin to form a stable complex with the receptor (Oakley et al. 2000; Simaan et al. 2005). The scaffolding function of arrestins, which recruit multiple binding partners to each receptor/arrestin complex, will induce conformational rearrangement of the arrestin, therefore, regulating GPCRs trafficking. The endosomal complex identity is known to depend on the type of binding proteins associated to the arrestin, but ‘how’ does the arrestin choose its binding partners remains an open question.

5.4. Beta-arrestins: Potential target for drug discovery

Besides trafficking and signaling functions, several studies showed that β -arrestin is involved in many physiological processes of the human body, including embryological

development, cardiovascular functions, skeletal remodeling and metabolism. Beta-arrestins signaling functions were identified using three different strategies: First is generating arrestin knockout mice models; Second is introducing GPCRs mutations to block G protein coupling or arrestin recruitment; Third is using bias ligands that direct receptor signaling through the arrestin pathways over the G protein activation or *vice versa* (Luttrell and Gesty-Palmer 2010). For instance, deletion of both β -arrestins in mice results in embryonic death, whereas the loss of either β -arrestin-1 or β -arrestin-2 does not cause lethality but instead shows distinct phenotypic outcomes mainly due to impairments in GPCRs desensitization and internalization. Such effect will promote receptor accumulation at the plasma membrane, leading to an increase in the G protein activation. An example of this is a study showing that the activation of μ opioid receptors (μ OR) in β -arrestin-2 knockout mice potentiates the morphine-induced analgesia when compared to control mice suggesting that, *in vivo*, β -arrestin-2 plays an important role in regulating GPCRs signaling functions (Bohn, et al. 2002; Bohn, et al. 1999). In addition to β -arrestin-null mice, receptor mutations have also revealed several physiological features of β -arrestins signaling role. For instance, introducing mutations in the conserved DRY motif of the second intracellular loop, such as D125G/R126G/Y127A/M134A (DRYM/GGAA) or D125A/R126A (DRY/AAY) of the AT1R hampered the G-protein coupling to the receptor without altering arrestin recruitment (Gaborik, et al. 2003; Seta, et al. 2002). An example of this is an *in vivo* study showing that cardiac-specific overexpression of such AT1R mutant results in cardiomyocyte hypertrophy as well as bradycardia (Zhai, et al. 2005). Furthermore, subsequent studies using bias ligands have then provided insights on the arrestin

signaling impact on the cardiovascular system. For instance, studies using the Sar¹-Ile⁴-Ile⁸-AngII (SII) and the TRV120027, which block G protein activation while inducing β -arrestin-dependent signaling, have shown to increase cardiomyocyte contractility and improve cardiac performances in rats (Hostrup et al. 2012; Violin, et al. 2010). Such cardioprotective effects were also observed with carvedilol, a bias ligand known to induce β 1AR-mediated MAPK signaling pathways via EGFR transactivation in a β -arrestin dependent manner (Noma et al. 2007). Other than heart diseases, several reports also showed that β -arrestins are involved in various endocrine pathologies, such as diabetes (Dalle, et al. 2011; Sonoda, et al. 2008), Alzheimer disease (Thathiah, et al. 2013), psychosis (Chen, et al. 2012), osteoporosis via the parathyroid hormone receptor (Gesty-Palmer, et al. 2009) and schizophrenia via the dopamine D2 receptor (Urban, et al. 2007). These evidence have defined β -arrestin signaling as a potential target for drug discovery. On the other hand, it is important to note that inhibiting the arrestin signaling pathway does not always provide positive biological outcomes. For example, a selective biased ligand TRV130 of the μ OR was shown to induce G protein-mediated signaling without β -arrestin recruitment nor receptor internalization. This peptide showed a higher analgesic efficacy, lower respiratory suppression and less gastrointestinal dysfunction when compared to morphine (DeWire, et al. 2013).

Therefore, the best proposed therapeutic approach is yet to selectively induce or inhibit a ‘protein-protein’ interaction. For that, the arrestin seems to be the perfect target because of several properties: First is the signaling scaffold function, where β -arrestin-mediated biological responses depends on the interaction between the arrestin and other binding partners such as receptors and signaling effectors that determine the endosomal

complex signature. Second is the wide range of cellular signaling mediated by the arrestin upon agonist stimulation. Disrupting arrestin/receptor complexes could impair multiple signaling pathways involved in various cellular responses such as apoptosis, growth, proliferation and cell survival. Third is the expression of only two subtypes of non visual arrestins in mammals cells, which makes it easier to target all the arrestins in the cell in order to modulate receptor trafficking and signaling.

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Chapter 2

Rationale and Objectives

The main objective of this thesis is to develop a better understanding of the functional role of β -arrestins in GPCRs trafficking.

Beta-arrestins are multifunctional adaptors that interact with agonist-activated G protein-coupled receptors and terminate their signal transduction, commonly, through clathrin-mediated internalization. Once localized in endosomal compartments, β -arrestin also serves as a scaffolding protein, and recruits various signaling effectors such as Raf-1, MEK1 and ERK1/2. However, the signaling function of β -arrestins in regulating receptor/arrestin endosomal complexes remains unclear. Since we have already demonstrated that ERK1/2 is recruited to B2R/ β -arrestin-2 endosomal complexes, and given the fact that β -arrestins scaffolding function is important in modulating GPCRs internalization, we thus hypothesized that MAPK targets β -arrestin-2 in the endosomes and this process is responsible for regulating the trafficking of B2R.

The objectives of the first study are:

- 1) Determine the effect of MAPK on B2R/ β -arrestin-2 endosomal interactions.
- 2) Address the role of MAPK in B2R trafficking.
- 3) Identify the target site of ERK1/2 on β -arrestin-2.
- 4) Test the effect of the ERK1/2 target site on GPCRs trafficking.

Besides the important role of β -arrestins in GPCRs endocytosis, several other functions of arrestins have been identified over the years. However, most studies that investigated the effect of β -arrestin on cell signaling and/or biological responses have been using genetic tools (dominant negative forms of arrestins; siRNA knock-down), but no selective inhibitor of β -arrestin was yet developed.

Therefore, the objectives of the second study are:

- 1) Identify a new pharmacological inhibitor of β -arrestin by using a virtual screen based on the crystal structure of β -arrestin-1.
- 2) Test the effect of the new compound on:
 - a- Receptor endocytosis and recycling.
 - b- Receptor/ β -arrestin endosomal interactions.
 - c- Arrestin-dependent MAPK activation.

Several lines of evidence have shown that the binding of β -arrestin to the phosphorylated C-tail of GPCRs induces conformational rearrangements in several components of the arrestin. Amongst the arrestins domains, the polar core is one of the key interaction that maintains the arrestin at a high affinity binding state with the receptor. Therefore, identifying a selective compound that binds the polar core, competes with the receptor C-tail and blocks the interaction between the arrestin and the receptor consists of a useful tool to study β -arrestins several functions on GPCRs trafficking and signaling.

Connecting text

Background: Many previous studies described β -arrestins as signaling adaptors and investigated the effect of β -arrestins on MAPK signaling pathways. However, no reports have yet showed the role of endosomal signaling effectors in the regulation of β -arrestin/GPCR complex formation. Therefore, we sought to determine the effect of MAPK on the regulation of the B2R/ β -arrestin endosomal interactions. To do so, we first decided to evaluate the effect of MAPK on the lifetime of B2R/ β -arrestin-2 complexes in endosomes using FRAP technique; then, investigate the role of MAPK in B2R endosomal trafficking and finally identify the MAPK target site on β -arrestin-2 and determine if such putative site is important for regulating the interaction between β -arrestin-2 and GPCRs.

Results: The following study reveals a specific mechanism where the endosomal GPCR/ β -arrestin/MAPK signaling complex plays a positive feedback role in regulating receptors trafficking and intracellular signaling.

Conclusion: Differential MAPK-dependent regulation of endosomal complexes exists amongst β -arrestin subtypes and species.

Significance: Such divergent mode of regulation may help understanding the physiological role of the endosomal GPCR/ β -arrestins signaling axis.

Chapter 3

Differential Regulation of Endosomal GPCR/Beta-arrestin Complexes and Trafficking by MAPK

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Abstract

Beta-arrestins are signaling adaptors that bind to agonist-occupied G protein-coupled receptors (GPCRs) and target them for endocytosis; however, the mechanisms regulating receptor/ β -arrestin complexes and trafficking in endosomes, remain ill defined. Here we show, in live cells, differential dynamic regulation of endosomal bradykinin B2 receptor (B2R) complexes with either β -arrestin-1 or -2. We find a novel role for MAPK in the B2R/ β -arrestin-2 complex formation, receptor trafficking and signaling mediated by an ERK1/2 regulatory motif in the hinge domain of the rat β -arrestin-2 (PET¹⁷⁸P), but not rat β -arrestin-1 (PER¹⁷⁷P). While the ERK1/2 regulatory motif is conserved between rat and mouse β -arrestin-2, it is surprisingly not conserved in human β -arrestin-2 (PEK¹⁷⁸P). However, mutation of Lysine 178 to Threonine is sufficient to confer MAPK sensitivity to the human β -arrestin-2. Furthermore, substitution for a phosphomimetic residue in both the rat and the human β -arrestin-2 (T/K¹⁷⁸D) significantly stabilizes B2R/ β -arrestin complexes in endosomes, delays receptor recycling to the plasma membrane and maintains intracellular MAPK signaling. Similarly, the endosomal trafficking of β 2-adrenergic, angiotensin II type 1 and vasopressin V2 receptors was altered by the β -arrestin-2 T¹⁷⁸D mutant. Our findings unveil a novel subtype specific mode of MAPK-dependent regulation of β -arrestins in intracellular trafficking and signaling of GPCRs, and suggest differential endosomal receptor/ β -arrestin-2 signaling roles amongst species.

Introduction

Heptahelical receptors such as G protein-coupled receptors (GPCRs) are implicated in almost all physiological processes including neurotransmission, metabolism, cardiovascular and hormonal functions (1-3). Levels of GPCRs at the plasma membrane and their functional coupling to downstream signaling pathways, which contribute to these physiological responses are also intricately linked to β -arrestins' functions.

Beta-arrestins are cytosolic proteins that terminate G protein-dependent signaling and act both as endocytic and signaling adaptors (4,5). The arrestin family includes four members encoded by different genes (6): two visual arrestins (arrestin 1 and 4), which are confined to photoreceptors, and two non-visual ones, β -arrestin-1 and -2 (also commonly referred to as arrestin-2 and -3, respectively) that are expressed more ubiquitously and modulate the signaling and trafficking of many GPCRs. The current model for β -arrestins' roles first requires the phosphorylation of agonist-bound receptors by dedicated GPCR kinases, and their recruitment to the phosphorylated GPCRs (7,8). Beta-arrestins will then serve as adaptors to link GPCRs to components of the endocytic machinery like clathrin and its adaptor protein 2 (AP-2), allowing receptors to internalize (4,5,9). Whether β -arrestins traffic inside the cells and complex with some receptors into endosomes or whether they dissociate from them at the plasma membrane during endocytosis, have also been used as criteria to typify GPCRs into different classes (10). For instance, Class A receptors, which usually display higher affinity for β -arrestin-2 than for β -arrestin-1, form short-lived complexes with both β -arrestins at the plasma

membrane, internalize into endosomes without β -arrestins and are rapidly recycled to the plasma membrane. On the other hand, Class B receptors bind both β -arrestins with apparent similar affinities, form longer-lived complexes with β -arrestins into endosomes, and are generally retained for longer periods inside the cell, even if the ligand has been removed from the extracellular milieu. We have recently showed a hybrid trafficking behaviour of β -arrestin with the bradykinin B2 receptor (B2R), a member of the GPCR family involved in the regulation of the renal and vascular functions. Similarly to class B receptors, B2R internalizes with β -arrestins into endosomes upon agonist stimulation (11). However, these complexes are short-lived in endosomes, and receptors rapidly recycle back to the plasma membrane following agonist removal, hence more reminiscent of class A GPCRs in regards to such trafficking behaviour. Whether, and if so, how receptor/ β -arrestin complexes are regulated in endosomes for different GPCRs, and especially for B2R, still remains an open question.

Beta-arrestins also act as signaling adaptors. For example, they recruit Src kinases to receptors and assemble MAPK components such as Raf-1, MEK1 and ERK1/2 in endosomes, to promote signaling (12-14). Others and we have shown that the internalization of GPCRs through the clathrin pathway is also regulated by the scaffolding signaling functions of β -arrestins (15-18). For instance, phosphorylation of β -arrestin-1 by ERK1/2 in the C-terminal domain differentially modulates its ability to bind clathrin and AP-2, and impedes β 2-adrenergic receptor (β 2AR) internalization. The recruitment of Src kinase to receptor/ β -arrestin-2 complexes allows the phosphorylation of the β -subunit of AP-2 and dynamin, which permits the internalization of the angiotensin II type 1 (AT1R) and the β 2AR (16,19). Since we have shown that B2R/ β -

arrestin complexes recruit ERK1/2 (20), and given the important role of β -arrestins in scaffolding kinases to regulate GPCRs internalization, we sought to determine the extent to which MAPK participates in the β -arrestin-dependent trafficking of B2R. Because β -arrestin subtypes are highly conserved amongst species, it is often assumed that they share most cellular and physiological functions—despite that few studies have addressed such issues. Moreover, because the GPCR/ β -arrestins signaling axis can be a potential therapeutic target, as recently demonstrated in different animal models such as rodents, we also compared β -arrestin 1 and 2 from rat and human in the β -arrestin-dependent MAPK regulation of different GPCR trafficking.

Experimental Procedures

Material - DMSO was from Bioshop. PD98059 inhibitor was from Calbiochem. PD184352 and SP600125 inhibitors were from Sigma-Aldrich. Anti-HA antibody coupled to agarose beads and mouse anti-HA antibody (clone 12CA5) were purchased from Roche (Laval, Canada). The polyclonal antibody against the C-terminal domain of β -arrestins, BARR3978 was described elsewhere (21). Mouse monoclonal anti-phospho-ERK1/2 (T202/Y204) and rabbit polyclonal anti-total ERK1/2 were from Cell Signaling Technology (Danvers, MA). Anti-mouse and anti-rabbit HRP-conjugated IgG were from Sigma-Aldrich (St-Louis, MO) and the chemiluminescence lightening (ECL) was from Perkin-Elmer. MEM and DMEM were from Hyclone (Logan, UT). Fetal bovine serum (FBS), L-glutamine and gentamicin were purchased from Invitrogen (Carlsbad, CA) and zeocin was from Invivogen (San Diego, CA). Dithiobis succinimidyl propionate (DSP) was from Pierce (Rockford, IL). Phenylmethyl sulfonyl fluoride (PMSF), aprotinin, leupeptin and pepstatin were from Bioshop (Burlington, Canada).

Plasmids and constructs - Plasmids encoding HA-B2R, HA-AT1R, HA-V2R, HA- β 2AR, B2R-YFP, human β -arrestin-2-YFP, rat β -arrestin-1-YFP and β -arrestin-2-myc have been previously described elsewhere (20-23). Dynamin K⁴⁴A was described in Zhang et al. (24). Rat β -arrestin-2-YFP construct was cloned into pEYFP-N1 using HindIII and KpnI sites. The rat β -arrestin-2-YFP T¹⁷⁸K, T¹⁷⁸A and T¹⁷⁸D constructs were generated by PCR using a forward primer overlapping the HindIII site in 5'-prime and a reverse primer for introducing either a Lysine, an Alanine or an Aspartic acid residue at position 178, and for creating an internal ApaI site. Another PCR was also generated

using a forward primer overlapping the *Apal* site and a reverse primer overlapping the *KpnI* site. The vector pEYFP-N1 was cut with *HindIII* and *KpnI*, and the two PCR products of each construct were three-way ligated with the digested vector. A similar strategy was also employed for generating the human β -arrestin-2-YFP K¹⁷⁸T and K¹⁷⁸D, with the exception of that the PCR products were cloned into pEYFP-N1 cut with *HindIII* and *BamHI*. The rat β -arrestin-2 S²⁶⁵A/T²⁷⁷A was generated by overlapping PCR. First, a PCR fragment was generated using a forward primer overlapping the *HindIII* site in 5'-prime with a reverse primer overlapping the *BlpI*, and second one using a forward primer overlapping *BlpI* including both mutations (S²⁶⁵A/T²⁷⁷A) with a reverse primer overlapping the *KpnI* site. Using these PCR fragments as a template, another PCR product was generated using a forward primer overlapping the *HindIII* site in 5'-prime and a reverse primer overlapping the *KpnI* site. The vector pEYFP-N1 was cut with *HindIII* and *KpnI*, and the final PCR product was ligated into the digested vector. For generating the rat β -arrestin-2-T¹⁷⁸D myc-tagged construct, a 5'-prime fragment of the rat β -arrestin-2-YFP-T178D was cut with *HindIII* and *BlpI*, and three-way ligated with a *BlpI*/*Sall* 3'-prime fragment in the pCMV-3Tag-2A vector previously digested with *HindIII* and *Sall*. Human MEK1 cDNA was provided by Dr. J. Charron (Université Laval, Québec, Canada). For the Flag-tagged MEK construct (MEKWT), the cDNA was cloned into pCMV-Tag using the *BamHI*/*EcoRI* sites. The MEK K⁹⁷A-Flag (MEKDN) was generated by replacing a *BamHI*/*BspEI* fragment from the MEK-Flag construct with a PCR fragment generated with a T3 forward primer and a 3'-prime reverse primer overlapping the Lys 97 codon (substituting the Lys for an Ala residue) and the *BspEI*, cut with the same enzymes. Red fluorescent-fused proteins (RFP) of MEK were generated

from MEKWT-Flag and MEKDN-Flag sequences by PCR using a forward primer overlapping the HindIII site (omitting the Flag sequence) and a reverse primer overlapping the stop codon and creating a KpnI site. PCR products were then cloned in frame into mRFP-C1 cut with the same enzymes. All constructs were analyzed by DNA sequencing (Sequencing Service, Genome Quebec Innovation Centre, McGill University, QC, Canada).

Cell culture and transfection - Stable human embryonic kidney (HEK) 293 cells expressing the B2R receptor (HEK293-B2R cells) were generated by transfecting the HA-tagged B2R cloned into pcDNA3.1/Zeo(+) (Invitrogen™, Life Technologies, Carlsbad, CA). Cells were then selected in 0.7 µg/ml zeocin, and expression levels were validated from radio-ligand binding assays (see below). Expression levels of receptor were found to be around 250 fmol/mg of total cell protein (i.e. ≈75,000 receptors/cell). HEK293 cells were grown at 37 °C in 5% CO₂ in MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and gentamicin (100 µg/ml). Cells were seeded at densities of 4x10⁵ cells per 100 mm dish or at 1x10⁵ cells per 35 mm dish, and transfected using conventional calcium phosphate co-precipitation methods. COS-7 cells were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 100µg/ml gentamicin (Gibco™, Life Technologies, Carlsbad, CA). Transfection of COS-7 cells that were seeded in 100 mm dish at a density of 8x10⁵ cells per 100 mm dish or 8x10⁴ per well in a 24-well plate was performed using Lipofectamine 2000 (Invitrogen™, Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations using 1:2 ratio of DNA:Lipofectamine

in Opti-MEM (Gibco™, Life Technologies, Carlsbad, CA). All experiments were performed 48 hours post transfection.

Confocal microscopy experiments - HEK293 cells were seeded in 35 mm glass bottom dishes (MaTek corp., Ashland, MA) and transfected with HA-B2R and either the YFP-tagged rat β -arrestin-2 or mutants (T¹⁷⁸K, S²⁶⁵A/T²⁷⁷A, T¹⁷⁸A and T178D), or the YFP-tagged human β -arrestin-2 or mutants (K¹⁷⁸T and K¹⁷⁸D). For experiments with the HA-vasopressin V2 receptor (V2R), HA- β 2AR and HA-AT1R, receptors were individually expressed in HEK293 cells with either the YFP-tagged rat β -arrestin-2 or the T¹⁷⁸D mutant. For assessing MAPK activity, HEK293 cells were transfected with HA-B2R and the rat β -arrestin-2-YFP and either MEKWT-Flag or MEKDN-Flag. Forty-eight hours post-transfection, cells were serum starved for 30 min followed by stimulation with bradykinin, vasopressin, isoproterenol or angiotensin II (1 μ M) for 15 min. For pharmacological MEK inhibition, cells were treated with either DMSO (vehicle), PD98059 (20 μ M), PD184352 (20 μ M) or SP600125 (20 μ M) for 30 min before bradykinin stimulation and fluorescence recovery after photobleaching (FRAP) experiments were performed. FRAP was applied on endosomes containing β -arrestin-YFP as previously described (23,25). Briefly, endosomes from at least eleven to twenty four independent experiments were individually bleached, and fluorescence recovery was monitored over a period of 3 min with image acquisition every 30 s. Regions of interest (ROI) were bleached for 100 iterations using a 514 nm Argon laser set at 100% intensity on a Zeiss LSM-510 Meta laser scanning microscope with a 63x oil immersion lens. Images (1024x1024 pixels) were then collected using emission BP 530-600 nm filter. For detecting mRFP, a HeNe I laser was used with 543 nm excitation and LP 560 nm

emission filter sets. Fluorescence intensity data were converted into a linear regression plot to obtain maximal recovery as described previously (23,25) and expressed as percentage recovery (%) / time (s) in function of fluorescence recovery (%) using GraphPad Prism 4. In order to compare endosomal β -arrestin half-life recovery between experiments, maximal recovery after three minutes was fixed at 100% for each condition. Endosomes of similar size and intensity were used in all experiments.

For quantification of the number of endosomes containing B2R, HEK293 cells were seeded in 35 mm glass bottom dishes and transfected with B2R-YFP and β -arrestin-2-myc. Forty-eight hours post-transfection, cells were serum starved for 30 min, treated with either DMSO (vehicle) or PD98059 (20 μ M) for another 30 min, before bradykinin stimulation. After endosomal formation, as detected by the fluorescence of B2R-YFP, the total number of endosomes with diameters $>1 \mu$ m were counted using Image J software and expressed over cell surface. Ratios were calculated from three independent experiments, comparing DMSO to PD98059 treated cells.

For recycling experiments, HEK293 cells were seeded in 35 mm glass bottom dishes and transfected with B2R-YFP and either myc-tagged β -arrestin-2 or the T¹⁷⁸D mutant. For MEK inhibition, cells were also transfected with either MEKWT-mRFP or MEKDN-mRFP, and treating cells with either DMSO (vehicle), PD98059 (20 μ M) or PD184352 (20 μ M) for 30 min before bradykinin stimulation. Forty-eight hours post-transfection, cells were serum starved for 30 min followed by stimulation with bradykinin (1 μ M) for 15 min. After the appearance of B2R-YFP in endosomes, cells were washed 3 times with MEM medium to remove cell surface bound ligand, and images (1024x1024

pixels) of receptor were acquired for 40 min using conditions described for YFP detection.

Immunoprecipitation and western blot experiments - Co-immunoprecipitation of covalently cross-linked β -arrestin-2-YFP to HA-B2R were performed as previously described (11). Briefly, either COS-7 cells or HEK293-B2R cells were seeded at a density of 8×10^5 per 100 mm dish, then transfected the second day with HA-B2R and β -arrestin-2-YFP, and either MEKWT-Flag or MEKDN-Flag. Forty-eight hour post transfection, cells were serum starved as described previously and challenged with bradykinin (1 μ M) for 15 min. For pharmacological MEK inhibition, cells were first treated with either DMSO (vehicle) or PD98059 (20 μ M) for 30 min after being serum starved, and then stimulated with bradykinin (1 μ M) for 15 min. Agonist stimulation was stopped following the addition of dithiobis succinimidylpropionate (DSP) at a final concentration of 2 mM. Cells were then washed with PBS containing 50mM Tris-HCl to neutralize DSP, and lysed in a glycerol buffer containing 100 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 25 μ g/ml leupeptin, 2.5 μ g/ml aprotinin and 1 mM pepstatin. Cell lysates were cleared by centrifugation (14,000 rpm for 30 min), and the supernatants were incubated overnight with monoclonal anti-HA affinity agarose beads (Roche). Beads were then washed with glycerol buffer and denatured in Laemmli buffer. Samples were run on 10% SDS-PAGE, and western blotting was performed using the anti-HA (12CA5), anti- β -arrestin antibody (BARR3978) and anti-phospho-ERK1/2. Immunoreactivity was visualized by enhanced chemiluminescence according to the manufacturer's instructions (Perkin Elmer).

Radioligand binding assay - Ligand binding assay was performed as described previously with minor modifications (11). Briefly, COS-7 cells were seeded at a density of 8×10^5 cells in 100 mm dishes and transfected with HA-B2R and either β -arrestin-2 WT or T¹⁷⁸D. The next day, cells were transferred into 24-well plates at a density of 8×10^4 cells per well. In recycling experiments, after bradykinin stimulation (50 nM, 15 min), cells were washed with prewarmed medium to remove surface bound ligand, and then were kept at 37°C for the indicated time to allow receptor recycling. Incubation was immediately stopped by putting plates on ice, and cells were rapidly washed three times (5 min each time) with ice-cold acid buffer (50 mM sodium citrate, 90 mM NaCl, pH 4.0) followed by an one ice-cold PBS wash to remove both the unbound and the cell surface receptor bound agonist. Cells were then incubated with cold binding buffer (50 mM Tris-HCl, 1 mM captopril, 1mM phenanthroline-1,10, and 140 μ g/ml bacitracin, pH 7.4 at 4°C) and 1.28 nM of ¹²⁵I-[Tyr⁸]Bradykinin (1000ci/mmol) in a total volume of 0.5 mL, and kept overnight at 4°C. The next day, cells were washed three times with ice-cold PBS then solubilized with 0.5 mL of 0.5 M NaOH and 0.05% sodium dodecyl sulphate (wt/vol), and radioactivity was assessed using a gamma-counter. Nonspecific binding was measured in the presence of 1 μ M of unlabeled bradykinin. Percent of receptor internalization and recycling was calculated from the ratio of treated over non-treated conditions as describe previously (11).

MAPK assay - COS-7 cells were seeded in a 6-well plate at a density of 1×10^5 cells/well and transfected with HA-B2R and either the YFP-tagged rat β -arrestin-2 WT or the T¹⁷⁸D mutant, or with the YFP-tagged human β -arrestin-2 WT or the K¹⁷⁸D mutant. Forty-eight hours post-transfection, cells were serum starved for 30 min in DMEM

containing 20mM HEPES, and then left either untreated or treated with bradykinin (1 μ M) for the indicated time. Treatment was stopped on ice with cold PBS, and cells were solubilized in 2x laemmli buffer (250 mM Tris-HCl pH 6.8, 2% SDS (wt/vol), 10% glycerol (vol/vol), 0.01% bromophenol blue (wt/vol), and 5% β -mercaptoethanol (vol/vol)). Lysates were resolved on a 10% SDS-PAGE and analyzed by western blot using anti-phospho-ERK1/2, anti-total-ERK1/2, and anti- β -arrestin antibody (BARR3978).

Statistical analysis - Signals from western blots were determined by densitometry analysis using Image J, and are presented as the mean $\pm$ SEM of at least three independent experiments. Statistical analysis was performed using Graphpad Prism software using 't' tests or two-way ANOVA when appropriate. Bonferroni (comparison between all groups) post-tests were performed where necessary.

Results

MAPK regulates B2R/ β -arrestin-2 complexes in endosomes and receptor trafficking

Because β -arrestins act as signaling scaffolds, and because signaling through β -arrestins regulates endocytic complex assembly during the internalization of GPCRs (15,16,21), we first tested the role of MAPK signaling on B2R/ β -arrestin interaction and receptor trafficking. To this end, we expressed HA-tagged B2R with either the murine β -arrestin-1-YFP or β -arrestin-2-YFP in HEK-293 cells (Fig. 1). Cells were then treated with either DMSO (vehicle) or 20 μ M of MEK inhibitor PD98059, which inhibited ERK1/2 activation (Fig. 2A), before being challenged with bradykinin for 15 min to allow receptor internalization and the formation of B2R/ β -arrestins complexes in endosomes. To monitor the lifetime of B2R/ β -arrestin complexes in endosomes, we assessed in real time the fluorescence recovery of YFP labeled β -arrestins onto endosomes using a fluorescence recovery after photobleaching (FRAP) method. The recovery of β -arrestins-YFP onto the endosomes from a surrounding cytosolic pool is dependent on the strength of the interaction between the receptor and the bleached β -arrestins-YFP. It is inversely proportional to the stability of the complex (26), hence, if the interaction between β -arrestins and a receptor is labile, this exchange and the recovery of fluorescence will be faster, and *vice-versa*. Results showed that the fluorescence recovery of β -arrestin-1-YFP onto endosomes containing B2R occurred more rapidly than for β -arrestin-2-YFP (Fig. 1A and C, respectively). Indeed, quantification reveals that fluorescence half-time recovery was around three times faster for β -arrestin-1 than for β -arrestin-2 (42.90 ± 5.7 s and 123.40 ± 9.1 s, respectively; Fig. 2E).

Treating cells with the MEK inhibitor PD98059 significantly decreased the recovery time of β -arrestin-2 to endosomes by approximately 50% (123.40 ± 9.1 s to 65.99 ± 9.2 s; Figs. 1C and D, and 2E), while it had no significant effect on β -arrestin-1 recovery rates (Fig. 1A and B, and Fig. 2E). Similarly, expression of a dominant negative form of MEK-1 (MEKDN), which impedes receptor-mediated ERK1/2 activation, also significantly decreased by more than 40% the recovery time of β -arrestin-2-YFP (139.10 ± 13.0 s to 79.81 ± 7.2 s; Figs. 1E and F, and 2E). Other MAPK inhibitors were also tested on the endosomal complex formation. Results show that while the other MEK inhibitor PD184352 significantly decreased the recovery time of β -arrestin-2 on endosomes to half times similar to that of PD98059 (*i.e.* reduction of 45%; Fig. 2A, B and E), the JNK inhibitor SP600125 (Fig. 2C) had no significant effect on the receptor/ β -arrestin-2 interaction when compared to DMSO treated cells (Fig. 2D and E). Because ERK1/2 is found in a complex in endosomes with both β -arrestin-2 and B2R (20), we next wanted to confirm biochemically the effect of inhibiting MAPK on such complex formation. In cells challenged with bradykinin for 15 min, we observed a 40% decreased association between β -arrestin-2 and the immuno-precipitated B2R when cells were either treated with PD98059 (Fig. 3A and B) or transfected with MEKDN (Fig. 3C and D), as compared to control cells.

The dissociation of β -arrestins from B2R in endosomes is required for receptor recycling at the plasma membrane (11). We next sought to know how reducing the avidity of B2R/ β -arrestin-2 complex in the endosomes by inhibiting MAPK impacted recycling of receptors. HEK293 cells were transfected with B2R-YFP and myc-tagged β -arrestin-2, and cells were stimulated with bradykinin for 15 min to allow receptor

internalization. We observed the formation of many large endosomes containing B2R, whereas cells pre-treated with the PD98059 and challenged with bradykinin showed qualitatively smaller (Fig. 4A, middle panel), and quantitatively lesser endosomes containing receptors (Fig. 4B). When bradykinin was then removed from the milieu by washing to allow receptor recycling, we observed some regain of B2R signal at the plasma membrane in control conditions (DMSO treated cells; Fig. 4A, bottom panel), but the presence of receptor-labeled endosomes was still obvious. However, the disappearance of the fluorescence in the endosomes and the regain of receptor at plasma membrane were more noticeable in cells treated with either PD98059 or PD184352 (Fig. 4A, left and right bottom panels, respectively). Similarly, at 40 min post-ligand wash (Fig. 4C bottom panels) we observed that the disappearance of B2R from large endosomes and the recycling of the receptor at plasma membrane was more obvious in cells transfected with MEKDN than for control cells expressing wild type MEK. Interestingly, the remaining B2R in endosomes in MEKDN expressing cells following bradykinin removal, were found in smaller vesicles compared to those observed in the control cells. Taken together, these results suggest that the MEK-ERKs signaling axis plays an important role in regulating endosomal B2R/ β -arrestin-2 interaction and intracellular receptor trafficking.

Identification of a novel MAPK-dependent regulatory site in the hinge domain of β -arrestin-2

Because MAPK inhibition affected the endosomal interaction between B2R and β -arrestin-2, but not with β -arrestin-1, we sought to identify differences in their

sequences that would explain such distinct susceptibility. Analysis of β -arrestin-2 sequence identified three putative ERK phosphorylation sites in its sequence (*e.g.* S/TP: T¹⁷⁸, T²⁷⁷ and S²⁶⁵ with scores of 2.096, 1.096 and 0.352, respectively; www.scansite.mit.edu) (Fig. 5A). Only two of these residues are not conserved between the two subtypes (T¹⁷⁸, and S²⁶⁵), potentially excluding *de facto* T²⁷⁷ as the regulatory site. However, we substituted both T²⁷⁷ and S²⁶⁵ residues at once for Ala (S²⁶⁵A and T²⁷⁷A) to exclude any participation of these two sites with the lowest phosphorylation scores. The half-time recovery of the β -arrestin-2 mutant onto the endosomes was still significantly decreased by more than 60% with PD98059 treatment (Fig. 5C and E), suggesting that these residues are not the main targets of MAPK for regulating the receptor/ β -arrestin-2 interaction in endosomes. Thr¹⁷⁸ is part of the well conserved P-E-T/S-P motif and only found in the hinge domain of β -arrestin-2 (Fig. 5A and B) (27,28). Replacing Thr¹⁷⁸ for an Ala residue abolished the effect of PD98059 treatment on the receptor/ β -arrestin-2 complex half-life (Fig. 5D and E). Because Thr¹⁷⁸ seems to be the regulatory site, we next substituted it for an Asp residue to mimic a negative charge resulting for its phosphorylation, and then assessed the endosomal B2R/ β -arrestin-2 complex formation. FRAP results showed that introducing such negative charge in the β -arrestin-2 significantly increased the stability of the complex in endosomes because the recovery rates of the mutant was significantly slowed down, with half-time recovery that increased by more than 2 folds as compared to wild-type β -arrestin-2 (Fig. 6A, B and C). Such effect on the increased B2R/ β -arrestin-2 complex formation was also validated biochemically (Fig. 6D and E).

The hinge domain of β -arrestin-2 regulates endosomal interactions with other GPCRs and receptor trafficking

We next assessed to what extent mimicking Thr¹⁷⁸ phosphorylation affected the endosomal interaction of β -arrestin-2 with either class B GPCRs like the V2R and the AT1R, or a class A receptor, such as the β 2AR. Expressing β -arrestin-2 T¹⁷⁸D in cells significantly increased by more than 2.4 fold the half-life of complexes between V2R and β -arrestin-2 compared to wild type (Fig. 7A and B). The half-time recovery of β -arrestin-2 T¹⁷⁸D to V2R in endosomes became comparable to that of wild type β -arrestin-2 with AT1R. Moreover, the recovery rate of the phospho-mimic β -arrestin-2 mutant on the AT1R-containing endosomes was also increased by 1.4 fold (Fig. 7B). For the β 2AR, we observed the accumulation of β -arrestin-2 T¹⁷⁸D in endosomes following agonist stimulation (Fig. 7C). This contrasted with wild type β -arrestin, which, as we showed previously, can be recruited at the plasma membrane upon agonist activation of β 2AR, but does not internalize into endosome with the receptor (29,30). The endosomes containing β -arrestin-2 T¹⁷⁸D were smaller and more labile than the ones observed with class B receptors, which prevented us from performing FRAP quantifications. Together, our findings imply that the β -arrestin-2's hinge is important for its high affinity interaction with GPCRs.

We next investigated how β -arrestin-2 T¹⁷⁸D affected recycling of the B2R. Binding assays were performed in cells after bradykinin stimulation, and receptor levels at the plasma membrane were evaluated 5, 15 and 30 min after removing the ligand to allow receptor recycling. Results show that B2R recycling decreased by around 50% in conditions where β -arrestin-2 phospho-mimic mutant was expressed, as compared to wild

type β -arrestin-2 transfected cells (Fig. 8A). These findings were also supported by microscopy. We qualitatively observed less receptors trafficking back at the plasma membrane after ligand removal in cells expressing β -arrestin-2 T¹⁷⁸D than in those expressing wild-type β -arrestin-2 (Fig. 8B). Consistent with the lack of receptor recycling to the plasma membrane, the B2R/ β -arrestin-2 T¹⁷⁸D complexes migrated to larger perinuclear endosomes, while WT complexes were found in smaller recycling endosomes.

Differential endosomal signaling roles between human and rat β -arrestin-2

Threonine 178 in the hinge domain is present in many rodent β -arrestin-2 subtypes, but is replaced for a positive charge (Lys¹⁷⁸) in many other mammals like the human and bovine orthologs (Fig. 9A), suggesting that the regulation of endosomal β -arrestin/receptor complexes differs amongst species. We thus tested the effects of inhibiting MAPK on the lifetime interaction between human β -arrestin-2 and B2R. The half-time recovery of the human β -arrestin-2 (h β arr-2) on the endosome following photobleaching was 1.7 times faster than with rat β -arrestin-2 (r β arr-2) (73.55 $\pm$ 4.5s vs. 123.40 $\pm$ 9.1s, respectively), but 1.7 times slower than for rat β -arrestin-1 (r β arr-1) (73.55 $\pm$ 4.5s vs. 42.86 $\pm$ 5.7s, respectively) (Figs. 9A and F, and 2E). Moreover, recovery rates of the human β -arrestin-2 onto endosomes were not affected by MAPK inhibition (Fig. 9B and F). To further validate the role of this regulatory site on the β -arrestin/B2R, we generated a gain-of-function β -arrestin-2 by introducing a Threonine into the human ortholog (β -arrestin-2 K¹⁷⁸T); and *vice-versa*, generated a lost-of-function mutant by replacing the one in rat β -arrestin-2 by the analogous residue in the human β -arrestin-2

(T¹⁷⁸K), and assessed the dynamic regulation of the complex in endosomes by FRAP. Reconstituting the putative ERK phosphorylation site into the human β -arrestin-2 (K¹⁷⁸T) significantly increased its time of recovery onto endosomes by almost 35% as compared to WT (Fig. 9C and F), which half-time recovery was now comparable to that observed for the rat β -arrestin-2 (Fig. 2E). Similarly to the rat β -arrestin-2, endosomal recovery of human β -arrestin-2 K¹⁷⁸T became sensitive to MAPK inhibition, because its half-time recovery significantly decreased by more than 50% upon PD98059 treatment (Fig. 9C and F). On the other hand, endosomal half-time recovery of the rat β -arrestin-2 T¹⁷⁸K was similar to that observed for the wild-type human β -arrestin-2 (Fig. 9D and F), and was insensitive to MAPK inhibition, because treatment of cells with PD98059 did not significantly change its recovery time.

We next assessed how increasing the avidity of the B2R/ β -arrestin-2 complex impacted MAPK signaling itself. To this end we expressed either β -arrestin-2 T¹⁷⁸D or β -arrestin-2 K¹⁷⁸D in COS-7 cells, and assessed bradykinin-mediated ERK1/2 activation at different times. We used COS-7 cells, because they express low levels of endogenous β -arrestins and would allow better vetting the mutants' effects (31). In cells expressing either wild-type rat (Fig. 10A and B) or human (Fig. 10C and D) β -arrestin-2, we observed a biphasic activation of MAPK following bradykinin stimulation with an initial high response phase at 5 min, which is believed to be dependent on G proteins, followed by a lower, but more sustained β -arrestin-dependent ERK activation phase, which lasted up to 60 min. Expression of either rat β -arrestin-2 T¹⁷⁸D (Fig. 10A and B) or human β -arrestin-2 K¹⁷⁸D (Fig. 10C and D), significantly amplified (at 30 and 60 min) the agonist-mediated long-term MAPK phase. This sustained ERK1/2 activation phase was

dependent on B2R internalization, because the expression of a non-functional Dynamin (Dyn K⁴⁴A), which blocks GPCR internalization, prevented this response (Fig. 10E and F). These data suggest that the hinge domain of β -arrestin-2, which controls its high affinity binding to receptors, also plays an important role in the regulation of the B2R trafficking and signaling.

Discussion

We provide evidence for a new role of the MAPK signaling axis in the dynamic regulation of endosomal GPCR/ β -arrestin-2 complexes and receptor trafficking. We identified a putative ERK1/2 phosphorylation site, which was present in the hinge domain of murine β -arrestin-2, but not β -arrestin-1. Moreover, the findings that the ERK regulatory site is present only in the murine β -arrestin-2 species (Fig. 9A) not only underscores the differences that exist in the endocytic functions between the two non-visual arrestin subtypes, but also suggest evolutionary divergences in the roles of β -arrestin-2. The identification of a key residue in the hinge domain of β -arrestin-2 (Thr¹⁷⁸) also emphasizes the importance of this domain for regulating its interaction with GPCRs, which could be targeted to study the physiological roles of endosomal GPCR/ β -arrestin-2 complexes. Based on our results, we propose a model where the formation of a receptor/ β -arrestin/MAPK signaling complex plays an endosomal positive feedback role in the regulation of GPCRs trafficking and intracellular signaling (Fig. 11).

Beta-arrestins are implicated in both the trafficking and signaling of GPCRs (4,5,9). Here we have linked these two functions by providing evidence that signaling resulting from the scaffolding of MAPK with B2R/ β -arrestin complexes controls the intracellular trafficking of receptors through a critical Threonine residue in the hinge domain of β -arrestin-2. The role of MAPK in such regulatory mechanism is compelling for numerous reasons. First, we previously showed that following agonist stimulation of receptors, active ERK1/2 kinases are found in B2R/ β -arrestin-2 complexes (20). Second, inhibiting ERK activity, using either pharmacological inhibitors (PD98059 or PD184352)

of the upstream kinase MEK or expressing a dominant negative form of MEK-1, decreases the stability of B2R/ β -arrestin-2 complexes in endosomes, and favors the faster recycling of receptor to the plasma membrane. Third, and consistent with its phosphorylation by MAPK, the hinge domain of β -arrestin-2 contains an ERK1/2 phosphorylation motif (PET¹⁷⁸P) (32,33), as well as possesses a putative D-domain ERK1/2 docking site (K/R₂₋₃-X₁₋₆-L/I-X-L/I) (*e.g.* positions 161-169: KR-NSVR-L-I-I) (Fig. 5) (34). Fourth, we generate a gain-of-function mutant for the human β -arrestin-2, which possesses a Lys residue instead of the Thr¹⁷⁸. Substituting the Lys for a Thr residue in β -arrestin-2 rendered the receptor/ β -arrestin-2 complex sensitive to MAPK inhibition, contrarily to its wild-type counterpart that remained insensitive. Fifth, we found that the substitution of Thr¹⁷⁸ for a negatively charged residue both in the human and rat β -arrestin-2, which would mimic a phosphorylated state, had opposite effects on the B2R/ β -arrestin-2 complex stability and receptor trafficking compared to what was observed when inhibiting MAPK. Indeed, in such condition, we observed an increase in the endosomal lifetime of the B2R/ β -arrestin-2 complex, and a decrease in receptor recycling to the plasma membrane.

Interestingly, β -arrestin-1 lacks such ERK “phosphorylatable” motif in its hinge domain, but is nonetheless regulated by ERK1/2 phosphorylation for its endocytic function. In clathrin-coated vesicles at the plasma membrane, ERK1/2 phosphorylates β -arrestin-1 at Ser⁴¹² (absent in β -arrestin-2), with the consequence of regulating the binding to components of the coat (18). Our results suggest, however, that Ser⁴¹² is not a target for the regulation of β -arrestin-1 interaction with B2R in the endosomes, because inhibiting MAPK did not affect the dynamics of such complex formation. MAPK

phosphorylation of β -arrestins has thus been conserved as a mechanism for controlling GPCR internalization and receptor trafficking, but seems to have diversified in terms of its spatio-temporal roles (*e.g.* plasma membrane vs. endosomes).

How mechanistically the phosphorylation of, and/or the introduction of a negatively charged residue at position 178 in the hinge domain of β -arrestin-2 leads to increased affinity with activated receptors, is still unclear. However, crystallography studies and mutational analysis of different arrestins, which have provided some clues about the mechanics of GPCR/ β -arrestin binding, may also hint to how the hinge domain and its phosphorylation regulate such interaction (35,36). The binding of arrestins with activated, phosphorylated receptors follows a multistep conformational rearrangement process. At basal states, the N- and C- domains of β -arrestins (Fig. 5B), which form two β -sandwich folds that contain the receptor-binding elements in their concave polybasic surface, are maintained distant to each other in a “locked” and elongated arrangement by two key intra-molecular interactions. A first interaction forms a network of electrostatic interacting residues in the interdomain interface defined as the polar core. The second key interaction involves the three-element arrangement of the N-domain (*e.g.* two β -strands, and one α -helix) interacting with the C-tail of β -arrestins, which maintains the tail hidden in the core. Upon receptor activation, the engagement of the phosphorylated carboxyl-tail of GPCRs into arrestin neutralizes the positive charge of a key Arginine residue in the core domain (Arg¹⁷⁵ in arrestin and Arg¹⁷⁰ in β -arrestin-2). This will destabilize the polar core and the 3-element interactions and release the arrestin’s C-tail, which in turn will result in closing the N- and C- domains onto the receptor in a “clam-like” movement (37). The hinge domain that links the N- and C- domains thus would maintain this closed

state of arrestin, presumably through a new molecular rearrangement, which would stabilize furthermore the receptor/arrestin interaction. Introducing a negative charge at position 178 through phosphorylation could thus exert new electrostatic interactions of the hinge with other domains of β -arrestin-2 constraining it to maintain the closed arrangement of N- and C- domains on activated receptors, hence preserving a high affinity state of the receptor/ β -arrestin complex (Fig. 11). Alternatively, but not necessary mutually exclusive to the latter scenario, phosphorylation of Thr¹⁷⁸ and/or the introduction of a negative charge at this position, could facilitate the destabilization of the network interactions with Arg¹⁷⁰ in the core domain, allowing the more efficient “clam-like” closing of the N- and C- domains onto receptors. One would thus expect that under these conditions, β -arrestin-2 binds agonist-occupied receptors with higher affinity, and that at basal state if β -arrestin is prone to be phosphorylated in its hinge domain, receptors would also interact more efficiently with this “conformationally-primed” β -arrestin. Indeed, using the β -arrestin-2 T¹⁷⁸D mutant, we converted a class A trafficking behaviour to a class B one, such as in the case of the β 2AR, and increased the affinity of the receptor/ β -arrestin-2 complex in the endosome with class B GPCR, such as with the V2R and the AT1R. However, we did not detect the constitutive internalization and/or complex formation in endosomes of β -arrestin-2 T¹⁷⁸D with receptors. On the other hand, we did observe some ligand independent internalization of B2R and endosomal complex formation between receptors and β -arrestin-2 in conditions over-expressing a constitutively active form of MEK1¹. This suggests that β -arrestin-2 is preferentially targeted for phosphorylation when complexed with agonist-occupied receptors and that MAPK may also play other functions in the process of receptor internalization and

trafficking, than only increasing the affinity of β -arrestin for the receptor. Although more work is needed to fully understand the role of MAPK on the structural rearrangement of β -arrestin-2 our findings strongly support the importance of the hinge domain in the regulation of β -arrestin binding to GPCRs.

Our findings are also coherent with other studies showing that long-lived endosomal GPCR/ β -arrestin complexes facilitate continued intracellular MAPK signaling and affect receptor recycling at the plasma membrane (11,38). We found a direct correlation between the increased affinity of B2R/ β -arrestin-2 complexes in endosomes and the sustained MAPK signaling, as well as with the reduced kinetics of receptor recycling from endosomes back to the plasma membrane.

Our data not only highlight differences in the regulation of receptor/ β -arrestin complexes, but also that differences exist in the intrinsic affinity between β -arrestin-1 and β -arrestin-2, as well as amongst β -arrestin-2 from different species. For instance, the rat β -arrestin-2 had a greater affinity for B2R than the rat β -arrestin-1 and formed a tighter complex with B2R than human β -arrestin-2. These findings suggest that other determinants in each β -arrestins must also be involved in stabilizing the interaction with receptors. Other regulatory mechanisms than MAPK phosphorylation have also been implicated in regulating the affinity of β -arrestins with GPCRs. For example, β -arrestin-2 undergoes ubiquitination on two Lysines residues (Lys¹¹ and Lys¹²), which stabilizes its interaction with different receptors (39). Although we did not specifically assess whether Lys¹⁷⁸ in the human β -arrestin-2 was a target for ubiquitination, we do not believe that such mode of regulation is at play here, because we did not observe changes in the lifetime of endosomal B2R/ β -arrestins complexes when the Lysine was substituted for an

Alanine (K¹⁷⁸A)¹. From our data, we can draw an affinity hierarchy order for β -arrestins' interaction with receptors: rat β -arrestin-2 T¹⁷⁸D > human β -arrestin-2 K¹⁷⁸D > rat β -arrestin-2 $\approx$ human β -arrestin-2 K¹⁷⁸T > rat β -arrestin-2 T¹⁷⁸K $\approx$ human β -arrestin-2 > rat β -arrestin-1. We also propose that such characteristics could be used to study the role of receptor/ β -arrestin complexes in signaling and trafficking of GPCRs. However, an important aspect to consider when performing such studies in cells is the species and/or levels of endogenous β -arrestins present, as it is the case for example between HEK293 (*e.g.* human β -arrestin) and COS-7 cells (*e.g.* African green monkey, *Chlorocebus sabaeus*, Fig. 9A) because they may differentially compete and affect the responses mediated by a tagged β -arrestins exogenously expressed.

The hinge domain of β -arrestins is an exposed and molecularly flexible structure (37,40), which mechanistically provides an ideal localization for a phosphorylation regulatory site such as the one for MAPK. However, this motif is not found in all β -arrestin-2 species (Fig. 9A), and seems to have evolved differently amongst them. Indeed, the “phosphorylatable” threonine of the PXTTP motif is present both in mice and rat, but not all rodents, and in most other animals, a Lysine residue replaces it. Interestingly, the Threonine residue is also found in more distantly related animals (see, Fig. 9). Because the process of phosphorylation of proteins would require energy, this suggests that contrarily to other mammals, the rat and mice have preserved this ancestral mode of regulation (*e.g.* the increased endosomal β -arrestin-2 interaction with GPCR upon the hinge phosphorylation). In our limited phylogenetic analysis, we did not identify gain-of-function mutations (*e.g.* substitutions of Thr¹⁷⁸ for either Asp or Glu). This, however, was less surprising since non-synonymous mutations would require the spontaneous mutation

of at least two nucleotides for converting a Threonine (or a Lysine) into either an Aspartic or Glutamic acids, contrarily to only one for generating a Threonine from a Lysine (e.g. ACA→AAA, respectively), as it is the case here for the difference between the rat and the human β -arrestin-2. Moreover, the location of this regulatory site in rat β -arrestin-2 must also be critical, because its position did not change, as it often occurs in evolutionary processes between species for post-translational modified sites (e.g. phosphosites or ubiquitination ones) contained in disordered and/or flexible regions of proteins (41).

Our findings also open questions about the species-specific β -arrestin-dependent MAPK regulation of receptor trafficking and signaling on the physiological outcomes. Although the reasons remain unclear, regulating the interaction between GPCRs and β -arrestin-2 in endosomes, hence controlling some aspects of receptor signaling—e.g. at the plasma membrane by preventing receptor recycling and/or favoring the signaling inside the cell—might play differential physiological functions in rodent vs. human. New evidence suggests that the GPCR/ β -arrestin signaling axis may also be an important therapeutic target. Indeed, recent findings have shown that a biased ligand of AT1R favoring the GPCR/ β -arrestin-2 signaling complex is cardio-protective in rodents (42-44). The extend to which Thr¹⁷⁸ phosphorylation of β -arrestin-2 and the sustained endosomal AT1R/ β -arrestin signaling to MAPKs contribute to these effects are however still unclear. On the other hand, if such mechanisms is involved, it would only presumably contribute partially to this effect, because similar cardio-protective roles of the AT1R biased ligand have also been reported in dogs, whose β -arrestin-2, like in human, share a Lys residue at this position (Fig. 9) (45). Notwithstanding this latter

possibility, the existence of differential MAPK-dependent regulation of receptor/ β -arrestin signaling complexes for GPCRs should be considered when studying their physiological and pathophysiological roles in different animal models, as it may provide a therapeutic rationale for targeting such endosomal signaling complexes.

In summary, we provide here the first direct evidence for a role of MAPK in the endosomal trafficking of GPCRs. We identified a new regulatory site in the hinge domain of β -arrestin-2, which diverged amongst different species, and when targeted by phosphorylation not only increases the lifetime of receptor/ β -arrestin complex in endosomes, but also the intracellular signaling and trafficking of receptors. Taking advantage of this “regulatable” property of β -arrestin-2 should further our understanding of the role of the GPCR/ β -arrestin signaling axis in endosomes.

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Authors contribution - E.K. and S.A.L designed the study and E.K. performed the majority of the experiments. L.N. was involved in FRAP and MAPK experiments. E.K., Y.N. and M.S. performed the molecular biology. E.K. and S.A.L interpreted the data, and wrote the paper.

Footnotes

E.K. holds a studentship from the McGill Division of Endocrinology and Metabolism. This work was supported by Canadian Institutes of Health Research (CIHR) Operating Grants to S.A.L. (MOP-74603). S.A.L. held a “Chercheur Boursier Senior” scholarship from “Le Fonds de recherche du Québec - Santé” (FRQS).

¹ E. Khoury and S.A. Laporte, unpublished observations.

Abbreviations list

Adaptor protein 2:	AP-2
Angiotensin II type 1:	AT1R
Bradykinin B2 receptor:	B2R
β 2-adrenergic receptor:	β 2AR
Fluorescence recovery after photobleaching:	FRAP
G protein-coupled receptors:	GPCRs
MEK-1 dominant negative:	MEKDN
Vasopressin V2 receptor:	V2R

Figures and Legends

FIGURE 1

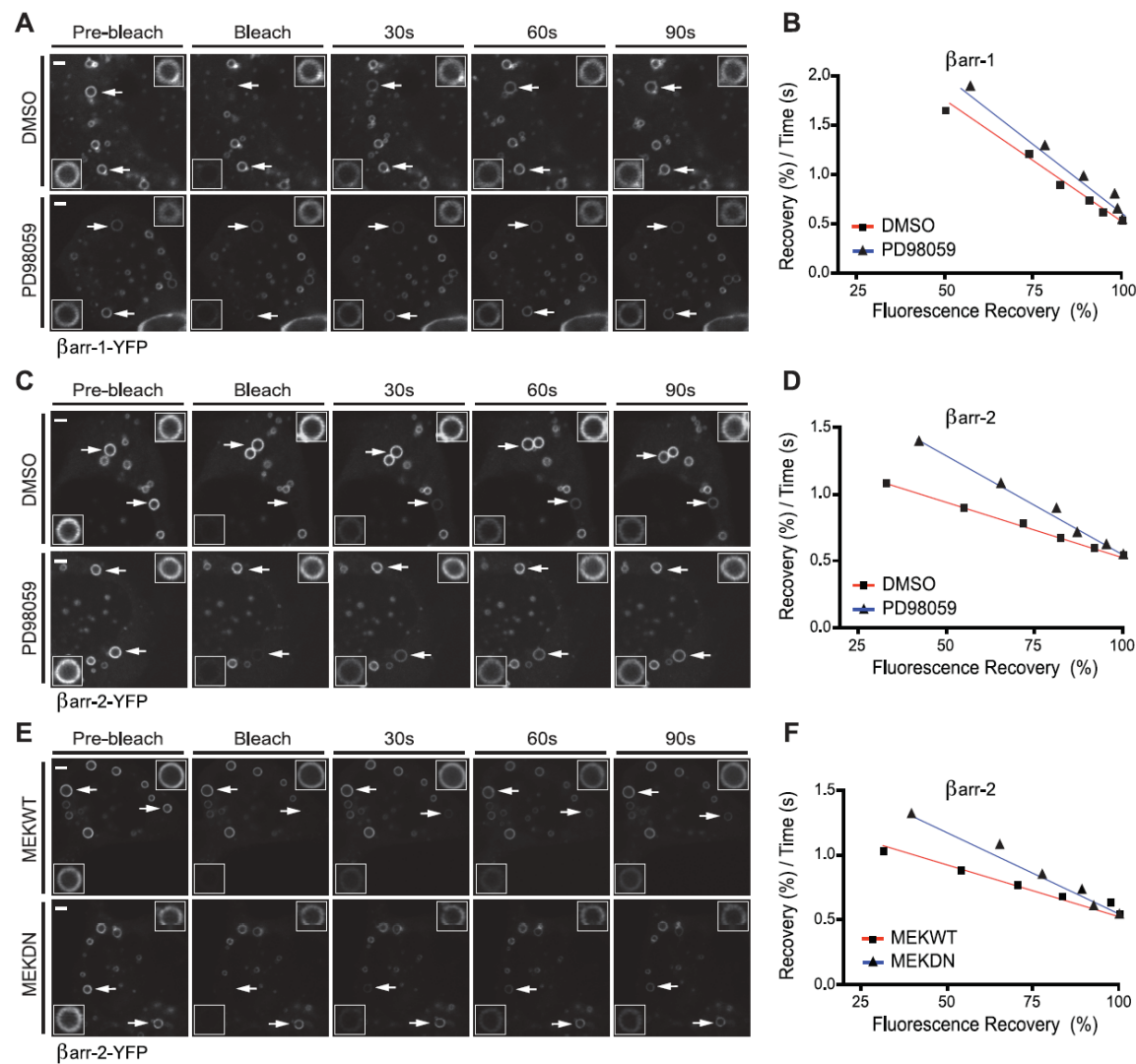


Figure 1. Inhibition of MAPK reduces the lifetime of B2R/ β -arrestin-2 complexes in endosomes. (A, C) Representative images of FRAP experiments on endosomes from HEK293 cells expressing HA-B2R and either (A), β -arrestin1-YFP (β arr-1-YFP) or (C), β -arrestin-2-YFP (β arr-2-YFP). Cells were either left untreated (DMSO) or treated with PD98059 (20 μ M, 30 min), before being challenged with bradykinin (1 μ M, 15min) as indicated in “Experimental Procedures”. Endosomes containing B2R and β -arrestins were selected (white arrows), and either bleached (bottom insets) or left unbleached (top insets). Fluorescence was then monitored over time every 30 seconds (30s, 60s, 90s). (E) Representative images of FRAP experiments on endosomes containing the β -arrestin-2-YFP, and transfected with either MEKWT or MEKDN. (B, D, F) Linear regression analysis of at least three independent experiments representing the recovery rates of β -arrestins onto endosomes containing B2R from panel A, C and E, respectively. Scale bars are 10 μ m.

FIGURE 2

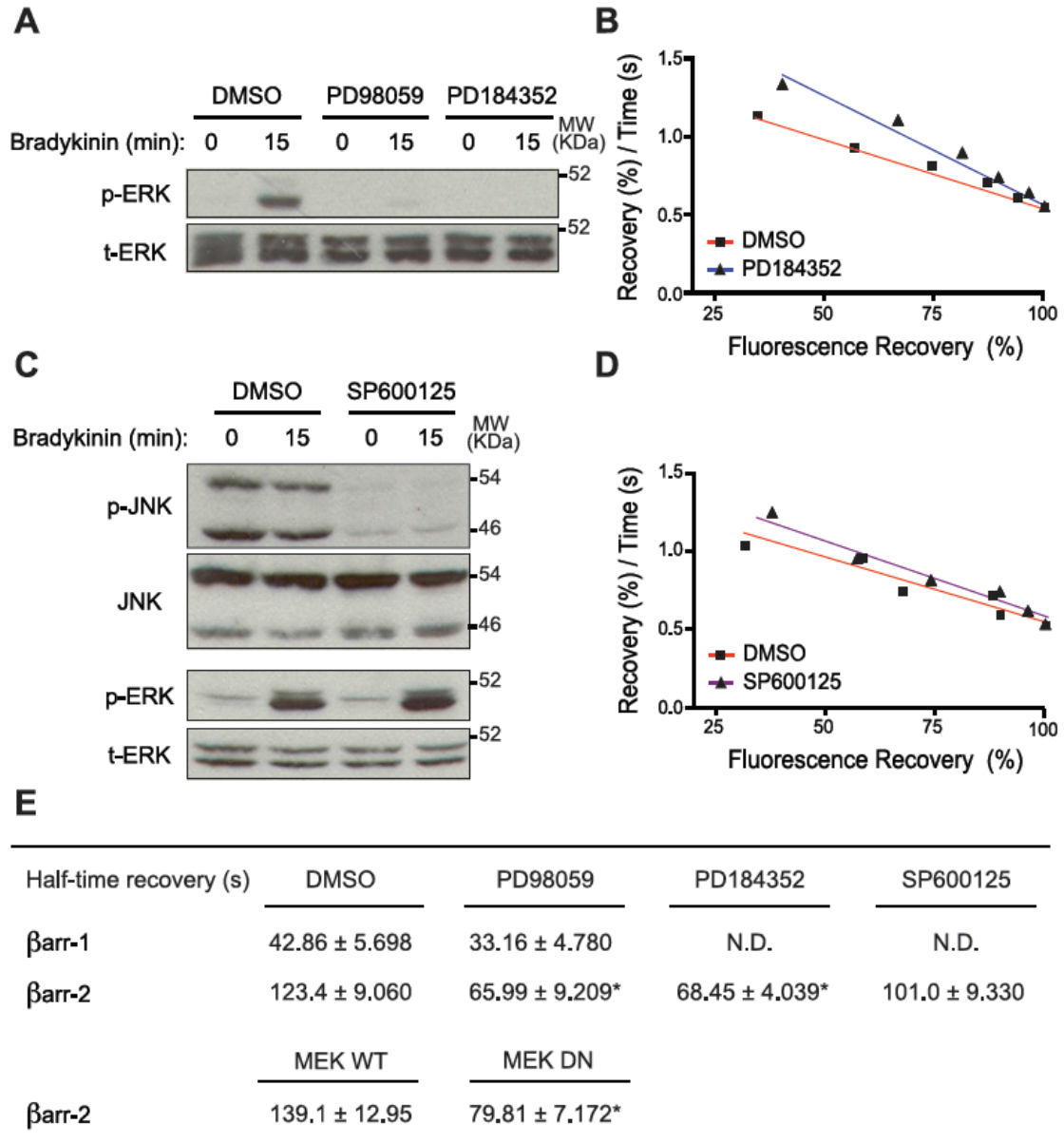


Figure 2. Inhibition of ERK1/2 activity decreases the life-time of B2R/ β -arrestin-2 complex in endosomes. (A, C) Shown are representative immuno-blots of ERK1/2 and JNK phosphorylation from HEK293 cells expressing HA-B2R and β -arrestin-2-YFP. Cells were either left untreated (DMSO), treated with (A) PD98059, PD184352 (20 μ M, 30 min) or (C) SP600125 (20 μ M, 30 min), then challenged with bradykinin (1 μ M) for 15 min. Lysates were immunoblotted for phosphorylated ERK (p-ERK), total ERK (t-ERK), phosphorylated JNK (p-JNK) and total JNK (JNK). Molecular markers of 46, 52 and 54 KDa are depicted. (B, D) FRAP analysis is presented as regression plots and was performed in HEK293 cells transiently transfected with HA-B2R and β -arrestin-2-YFP after 15 min of bradykinin stimulation (1 μ M), and either DMSO, (B) PD184352 (20 μ M, 30 min) or (D) SP600125 (20 μ M, 30 min) treatments as described in “Experimental Procedures”. (E) Shown are half-time recovery (s) data of β -arrestin-1 (β arr-1) and β -arrestin-2 (β arr-2) on endosomes containing B2R, calculated from the linear regression analysis presented in Figs. 1B, D, F and Fig. 2B, D. Statistical analysis were performed by using Student ‘t’ test; *, $p < 0.05$. Data are the mean $\pm$ SEM for 14-23 endosomes per condition.

FIGURE 3

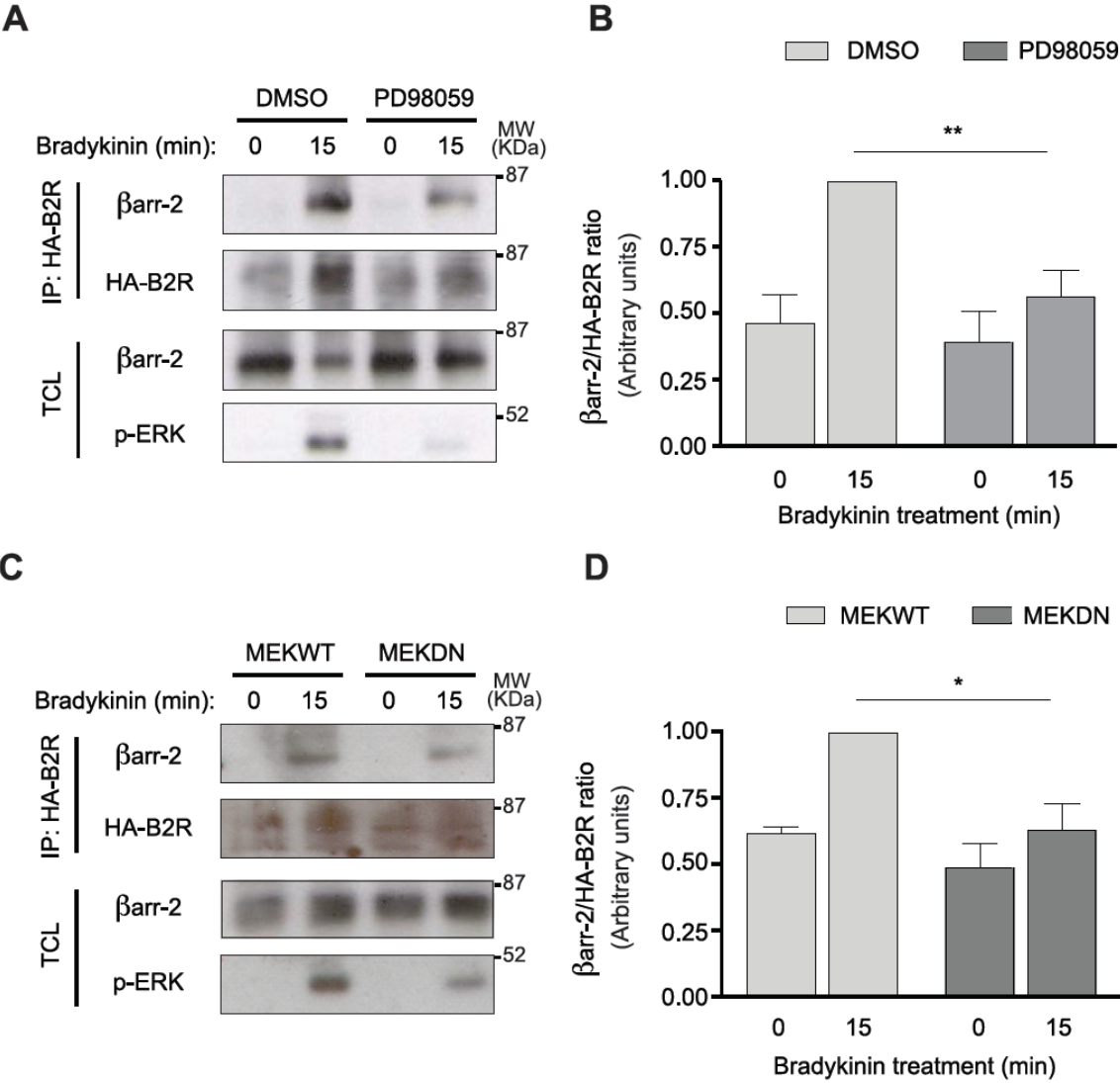


Figure 3. MAPK inhibition reduces B2R/ β -arrestin-2 complex formation. COS-7 cells expressing HA-B2R and β -arrestin-2 were (A) either treated with DMSO, with PD98059 (20 μ M, 30 min), or (C) transfected with MEKWT or MEKDN before being challenged with bradykinin (1 μ M, 15 min). Lysates from immuno-precipitated (IP) HA-B2R were analyzed by western blot using anti-HA (12CA5) and a β -arrestin antibody (BARR3978) for receptor and β -arrestin-2 detection, respectively. Total cell lysates (TCL) were blotted for β -arrestin-2 and p-ERK. Shown are representative blots of (A) five and (C) three independent experiments. Molecular markers of 52 and 87 KDa are presented. (B, D) Densitometry analysis of immunoprecipitation experiments. Data represent the relative expression of β -arrestin-2 normalized to the amounts of HA-B2R, as compared to the 15 min bradykinin stimulation, which is set at a 100% of (A) DMSO and (C) MEKWT conditions. Densitometry results are presented as the mean $\pm$ SEM. Statistical significance was analyzed by a two-way ANOVA followed by a Bonferroni post-test. *, $p < 0.05$, **, $p < 0.01$.

FIGURE 4

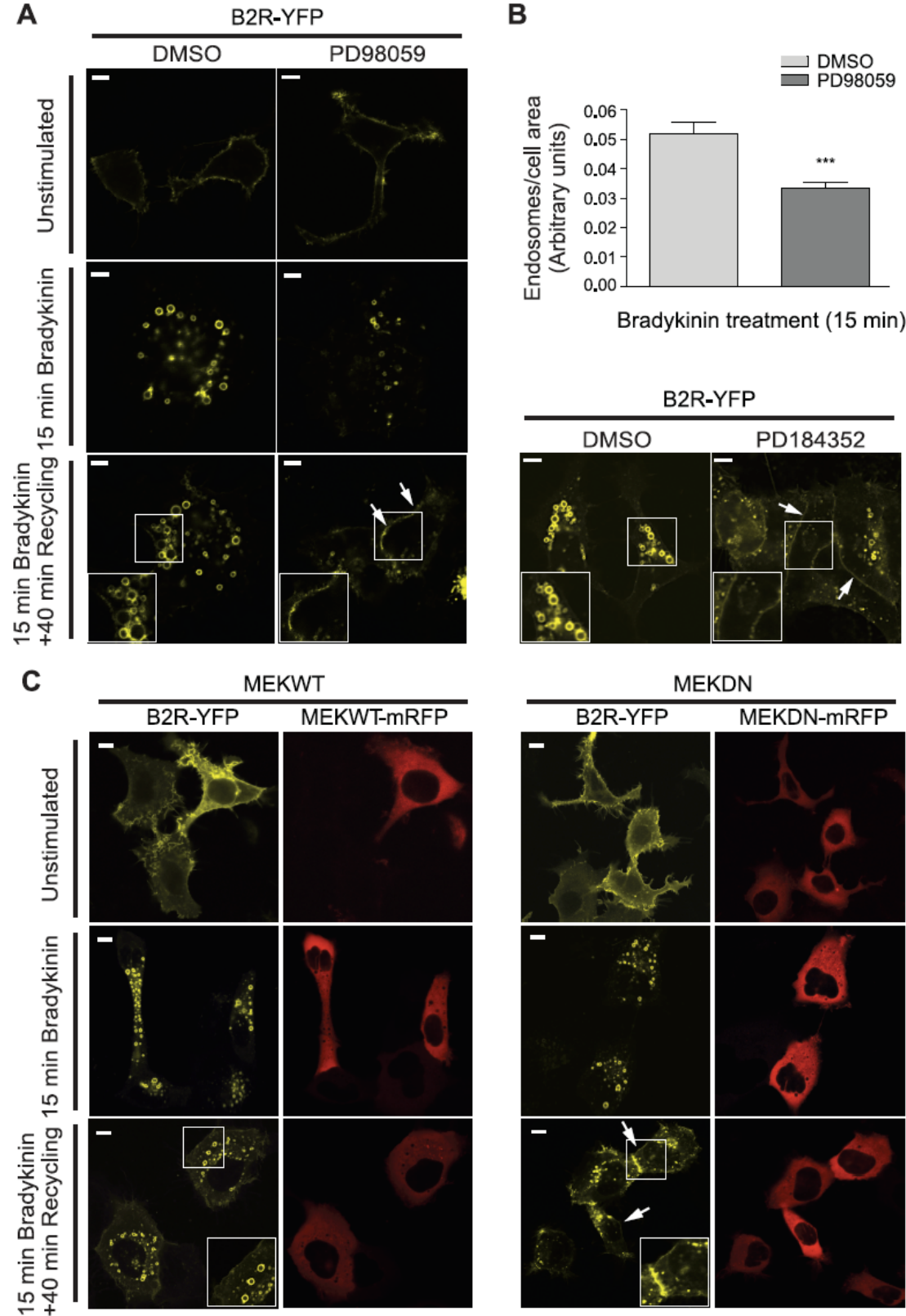


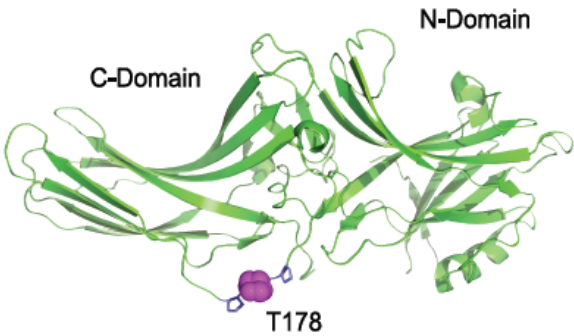
Figure 4. Inhibition of MAPK alters B2R trafficking. (A) Shown are representative confocal images of B2R-YFP internalization and receptor recycling in HEK293 cells. Cells expressing B2R-YFP and β -arrestin-2-myc were pretreated with either DMSO, PD98059 (20 μ M, 30 min; bottom left panel) or PD184352 (20 μ M, 30 min; bottom right panel), before being stimulated for 15 min with bradykinin (1 μ M) and imaged (middle panels), or washed to remove surface bound ligand and kept at 37⁰C to allow receptor recycling for 40 min (bottom panels). White arrows depict receptor recycling at the plasma membrane. Enlargement of B2R-YFP localization is shown in the left bottom insets of recycling conditions. Three independent experiments were performed collecting a total of 55-71 cells. (B) Quantification of endosomes (>1 μ m) after 15 min bradykinin stimulation. Results are from three independent experiments with the respective quantification of 80 and 96 cells for the different conditions, and are expressed as the number of endosomes over the cell surface area. Statistical significance was analyzed by Student “t” test; ***, $p < 0.001$. (C) HEK293 cells were transiently transfected with B2R-YFP and the β -arrestin-2-myc and either MEKWT-mRFP or MEKDN-mRFP and were treated as in (A). Shown are representative confocal images of B2R-YFP (yellow) and MEK-mRFP (red) depicting receptors trafficking to endosomes (middle panel) and their recycling to the plasma membrane (bottom panel). Results are representative images of three independent experiments surveying a total of 15-24 cells. Scale bars are 10 μ m.

FIGURE 5

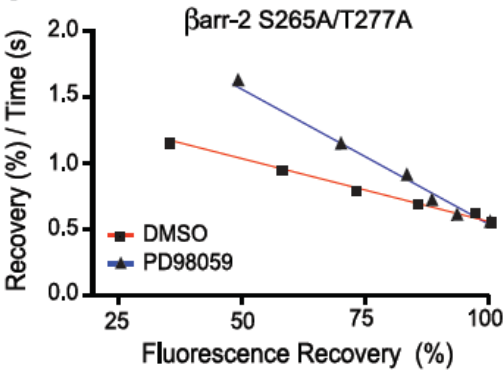
A

Beta-arrestin-1	KRNSVRLVIR KVQYAPERPG PQPTAETTRQ FLMSDKP_LH LEASLDKEIY YHGEPISVNV
Beta-arrestin-2	KRNSVRLIIR KVQFAPETPG PQPSAETTRH FLMSDRRLSH LEASLDKELY YHGEPLNVNV
	178
Beta-arrestin-1	HVTNNTNKT V KKIISVRQY ADICLFNTAQ YKCPVAMEEA DDTVAPSSTF CKVYTLTPFL
Beta-arrestin-2	HVTNNSAKT V KKIRVSVRQY ADICLFSTAQ YKCPVAQLEQ DDQVSPSSTF CKVYTIPTLL
	265 277

B



C



E

Half-time recovery (s)	DMSO	PD98059
β arr-2 S265A/T277A	109.1 $\pm$ 6.778	44.51 $\pm$ 4.171**
β arr-2 T178A	89.37 $\pm$ 1.032	111.9 $\pm$ 6.554

D

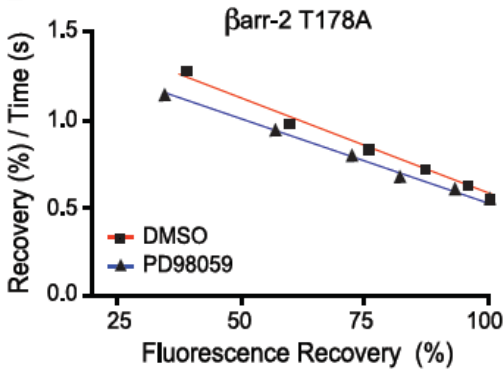


Figure 5. Role of Threonine 178 in B2R/ β -arrestin-2 endosomal complex interaction. (A) Amino acid sequence alignment is shown between β -arrestin-1 and β -arrestin-2. Potential MAPK motifs (S/TP) at position 178 (Thr), 265 (Ser) and 277 (Thr) in β -arrestin-2 are highlighted in bold and the putative D-domain ERK1/2 docking site (KRNSVRLIIR) is underlined in β -arrestin-2 sequence. (B) A representation of the 3D structure of the β -arrestin-2 using a cartoon visualization of the α -helices and the β -sheets by PyMol software. The ‘PET¹⁷⁸P’ motif is presented by amino acid lines structure. (C, D) FRAP analysis is presented as linear regression plots and was performed in HEK293 cells transiently transfected with HA-B2R and either the YFP-tagged β -arrestin-2 (C), S265A/T277A or (D) T178A. Cells were either treated with DMSO or with PD98059 (20 μ M, 30 min), before being challenged with bradykinin (1 μ M, 15min) as indicated in “Experimental Procedures”. (E) Shown are half-time recovery (s) data of β -arrestin-2 S²⁶⁵A/T²⁷⁷A and β -arrestin-2 T¹⁷⁸A mutants on B2R containing endosomes. Statistical analysis was performed by using Student ‘t’ test; **, p<0.01. Data are the mean $\pm$ SEM of three independent experiments, for 8-21 endosomes.

FIGURE 6

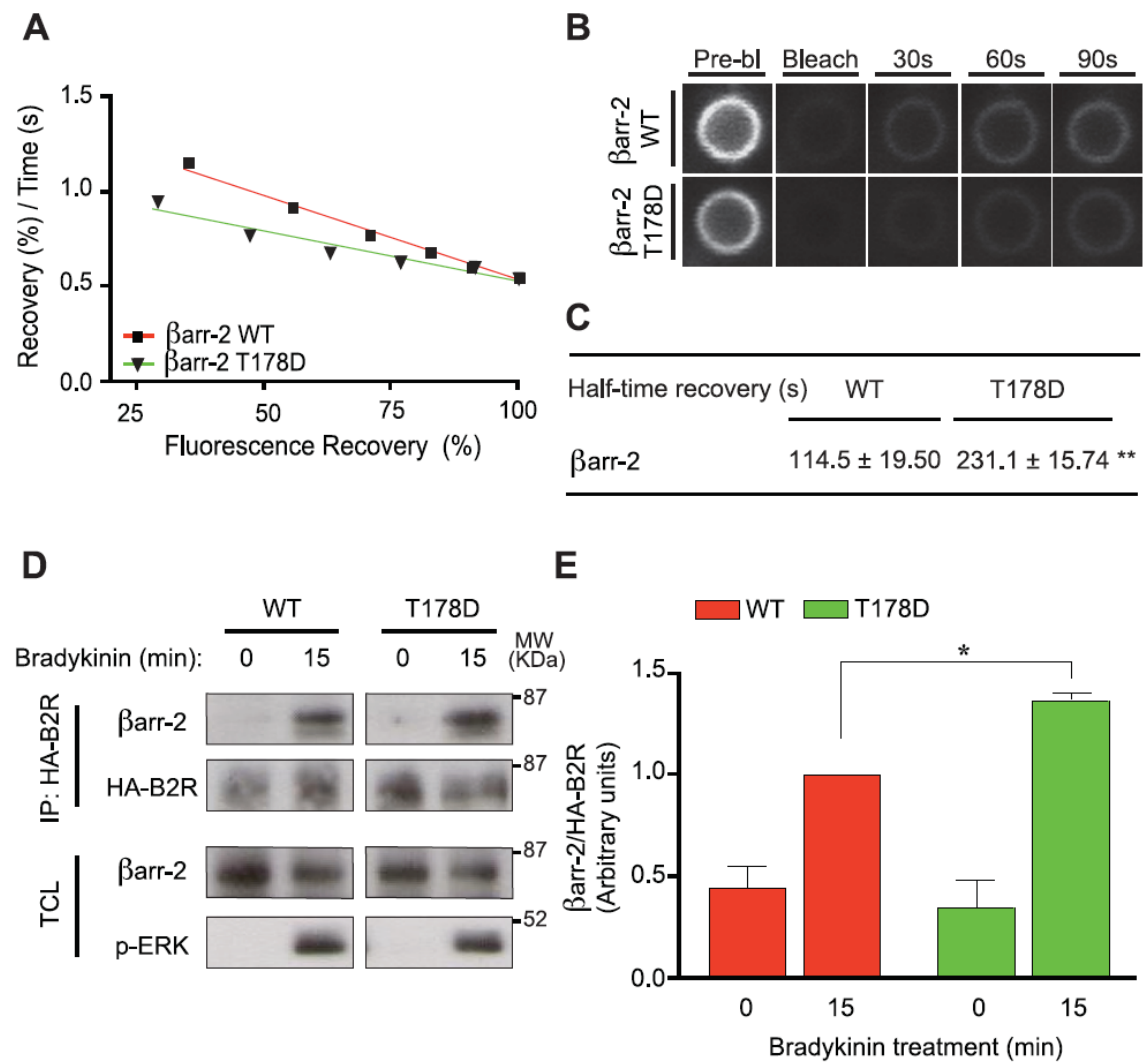


Figure 6. Aspartic acid substitution at position 178 of β -arrestin-2 promote B2R/ β -arrestin-2 endosomal complex formation. (A) Shown are regression plots from FRAP experiments performed on HEK293 cells transiently transfected with HA-B2R and either β -arrestin-2 WT (β arr-2 WT) or β -arrestin-2 T¹⁷⁸D (β arr-2 T178D) after 15 min of bradykinin stimulation (1 μ M) as described in “Experimental Procedures”. (B) Representative confocal images from FRAP experiments of agonist stimulated cells expressing HA-B2R and either β arr-2 WT or β arr-2 T¹⁷⁸D. Images are enlarged endosomes selected from 4-5 independent experiments assessing a total of 17 cells. (C) Shown are half-time recovery (s) data of β -arrestin-2 (β arr-2) WT and T¹⁷⁸D mutant on B2R containing endosomes, calculated from the linear regression analysis presented in A. Statistical analysis were performed by using Student ‘t’ test; **, $p < 0.01$. Data are the mean $\pm$ SEM of at least three independent experiments, for 17 endosomes. (D) HA-B2R stable cells expressing either β arr-2 WT or T178D mutant were left unstimulated or challenged with bradykinin (1 μ M) for 15 min. Lysates from immuno-precipitated (IP) B2R were analyzed by western blot using anti-HA (12CA5) and BARR3978 antibodies for receptor and β -arrestin-2 detection, respectively. Total cell lysates (TCL) were blotted for β -arrestin-2 and p-ERK. Shown are representative blots of three independent experiments. Molecular markers of 52 and 87 KDa are presented. (E) Densitometry analysis of immunoprecipitation experiments. Data represent the relative expression of β -arrestin-2 normalized to the amounts of B2R, as compared to the 15 min bradykinin stimulation, which is set at a 100% of the β arr-2 WT condition. Densitometry results are presented as the mean $\pm$ SEM. Statistical significance was analyzed by a two-way ANOVA followed by a Bonferroni post-test. *, $p < 0.05$.

FIGURE 7

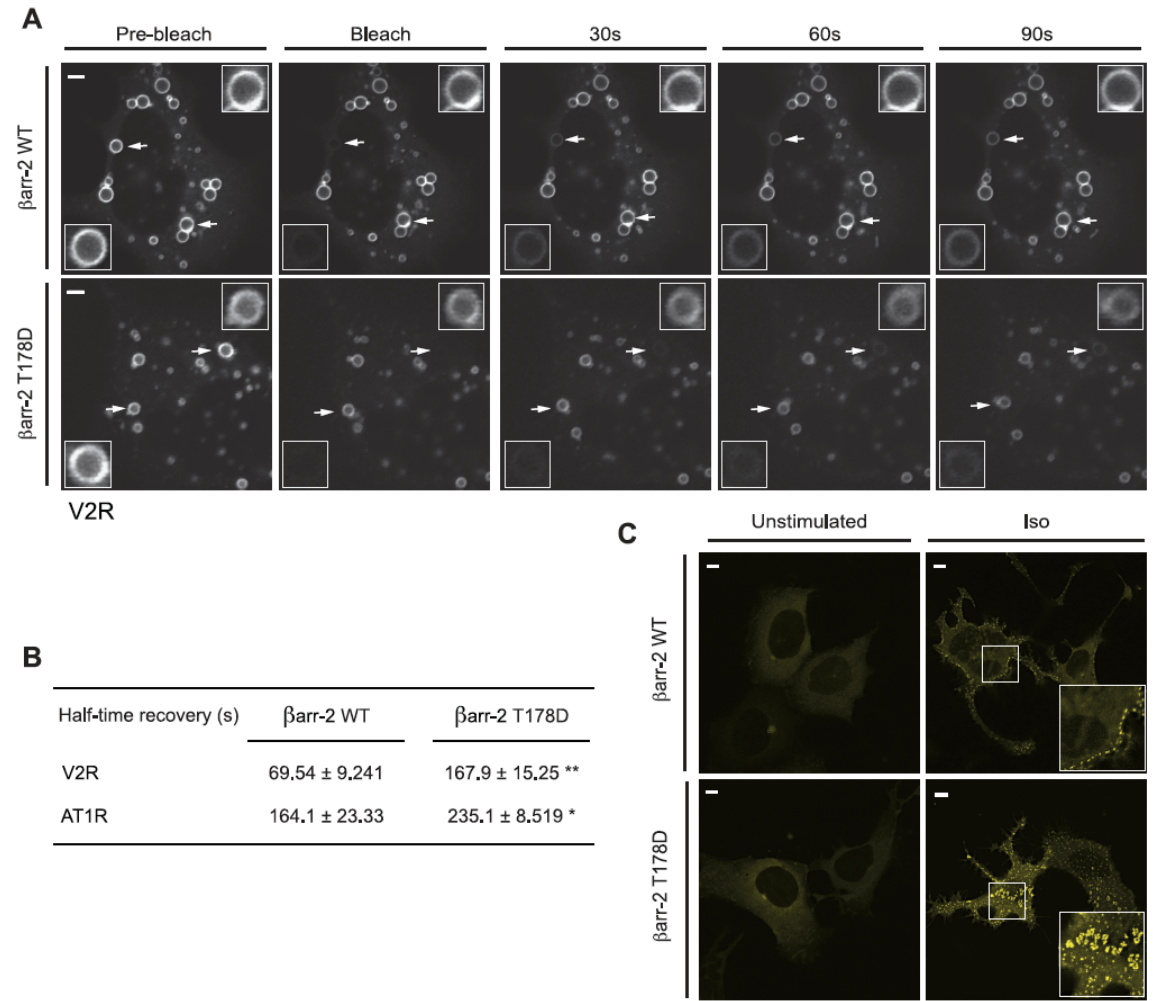
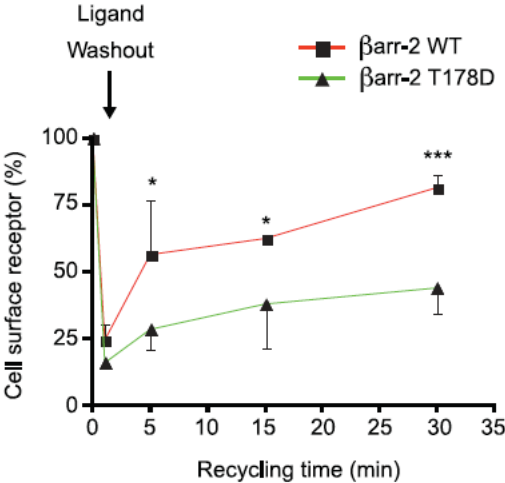


Figure 7. The introduction of a negative charge at position 178 of β -arrestin-2 stabilizes its interaction with V2R, AT1R and β 2AR. (A) Representative images of FRAP experiments on endosomes from HEK293 cells expressing HA-V2R and either YFP-tagged β -arrestin-2 (β arr-2) WT or β arr-2 T178D mutant. Cells were treated with vasopressin (1 μ M) for 15 min and FRAP analysis was performed as in Fig. 1. Endosomes containing receptors and β -arrestins were selected (white arrows), and either bleached (bottom insets) or left unbleached (top insets). (B) Shown are half-time recovery (s) data of β -arrestin-2 (β arr-2) WT and T¹⁷⁸D mutant endosomes from either HA-V2R or HA-AT1R transfected cells. Statistical analysis was performed by using Student 't' test; *, $p < 0.05$, **, $p < 0.01$. Data are the mean $\pm$ SEM of three independent experiments, for 11-17 endosomes. (C) Representative confocal images of HEK293 cells expressing the β 2AR with either YFP-tagged β arr-2 WT or β arr-2 T178D mutant, either stimulated with isoproterenol (1 μ M, right panels) for 15 minutes, or left unchallenged (left panels). An enlargement of the β -arrestin-2-YFP localization is shown in the bottom right insets of agonist-stimulated conditions. Scale bars are 10 μ m.

FIGURE 8

A



B

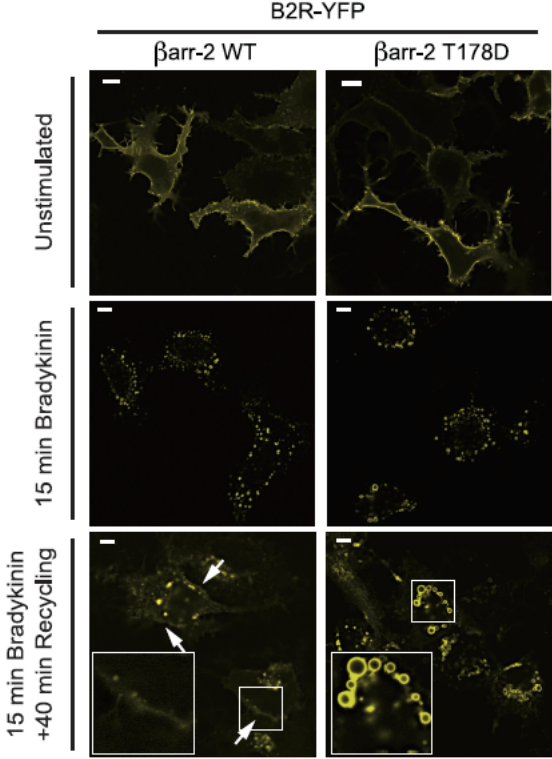


Figure 8. Introducing a negative charge at position 178 of β -arrestin-2 impedes B2R recycling. (A) Receptor recycling was assessed in COS-7 cells expressing the HA-B2R and either β -arrestin-2 (β arr-2) WT or β arr-2 T178D mutant as described in “Experimental Procedures”. Data are the mean $\pm$ SEM of three independent experiments. Statistical significance was analyzed by a two-way ANOVA followed by a Bonferroni post-test was performed. *, $p<0.05$, ***, $p<0.001$. (B) Representative confocal images of B2R internalization (middle panels), and recycling (bottom panels) in HEK293 cells as assessed in Fig.4. White arrows depict receptor recycling at the plasma membrane and the enlargement of B2R-YFP localization is shown in the left bottom insets of recycling conditions. Scale bars are 10 μ m.

FIGURE 9

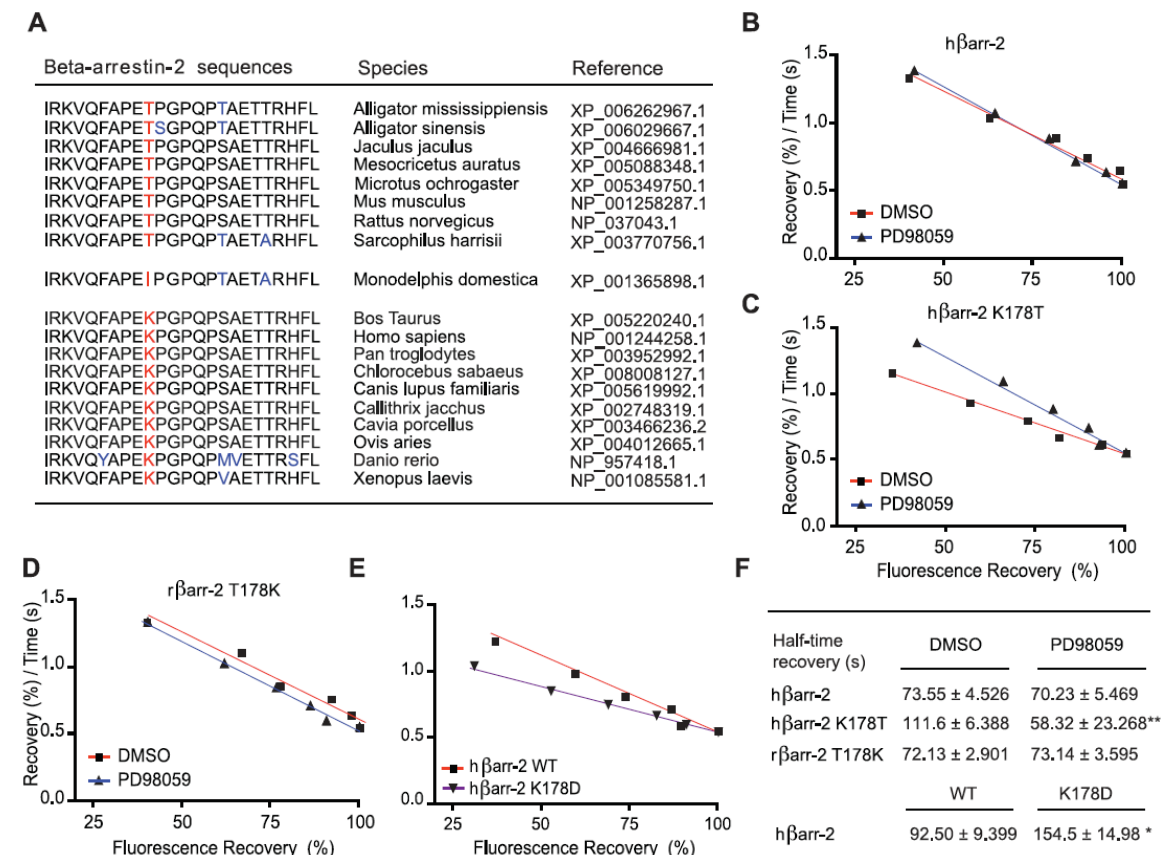


Figure 9. Position 178 in β -arrestin-2 regulates the interaction between B2R and arrestin in endosomes. (A) Interspecies alignment of the hinge domain of β -arrestin-2 sequences. Using BLAST (bl2seq), amino acid sequences were grouped by different ‘PEXP’ conserved motif. Red letters indicate the identified MAPK motif (PETP), which is converted to a lysine or an isoleucine amongst species and blue letters show the non-conserved amino acids. Other than homo sapiens, Lys178 is also conserved amongst different species such as, *Camelus ferus* (EPY84367.1), *Ceratotherium simum simum*

(XP_004433246.1), *Chinchilla lanigera* (XP_005399652.1), *Ailuropoda melanoleuca* (XP_002924519.1), *Tupaia chinensis* (XP_006151916.1), *Pan paniscus* (XP_003810257.1), *Sus scrofa* (ACF37110.1), *Trichechus manatus latirostris* (XP_004376131.1), *Otolemur garnettii* (XP_003791244.1), *Papio Anubis* (XP_003912182), *Saimiri boliviensis boliviensis* (XP_003931443.1), *Orcinus orca* (XP_004267017.1), *Dasypus novemcinctus* (XP_004475312.1), *Sorex araneus* (XP_004604974.1), *Octodon degus* (XP_004638414.1), *Mustela putorius furo* (XP_004760457.1), *Heterocephalus glaber* (XP_004901403.1), *Pantholops hodgsonii* (XP_005967979.1), *Vicugna pacos* (XP_006217626.1), *Nomascus leucogenys* (XP_004091595.1), *Odobenus rosmarus divergens* (XP_004398622.1), *Ochotona princeps* (XP_004594896.1), *Ictidomys tridecemlineatus* (XP_005337434.1), *Myotis lucifugus* (XP_006102837.1) (B, C, D, E) FRAP analysis is presented as regression plots and was performed in HEK293 cells transiently transfected with HA-B2R and either the YFP-tagged (B) human β -arrestin-2 (h β arr-2), (C) h β arr-2 K178T, (D) rat β -arrestin-2 (r β arr-2) T178K, or (E) h β arr-2 K178D after 15 min of bradykinin stimulation (1 μ M), and either DMSO or PD98059 (20 μ M, 30 min) treatments as described in Fig. 1 and in “Experimental Procedures”. (F) Shown are half-time recovery (s) of YFP-tagged h β arr-2 and r β arr-2 mutants, calculated from the linear regression analysis presented in panel B, C, D and E. Statistical analysis were performed by using Student ‘t’ test; *, $p < 0.05$, **, $p < 0.01$. Data are the mean $\pm$ SEM of at least three independent experiments for 14-24 endosomes.

FIGURE 10

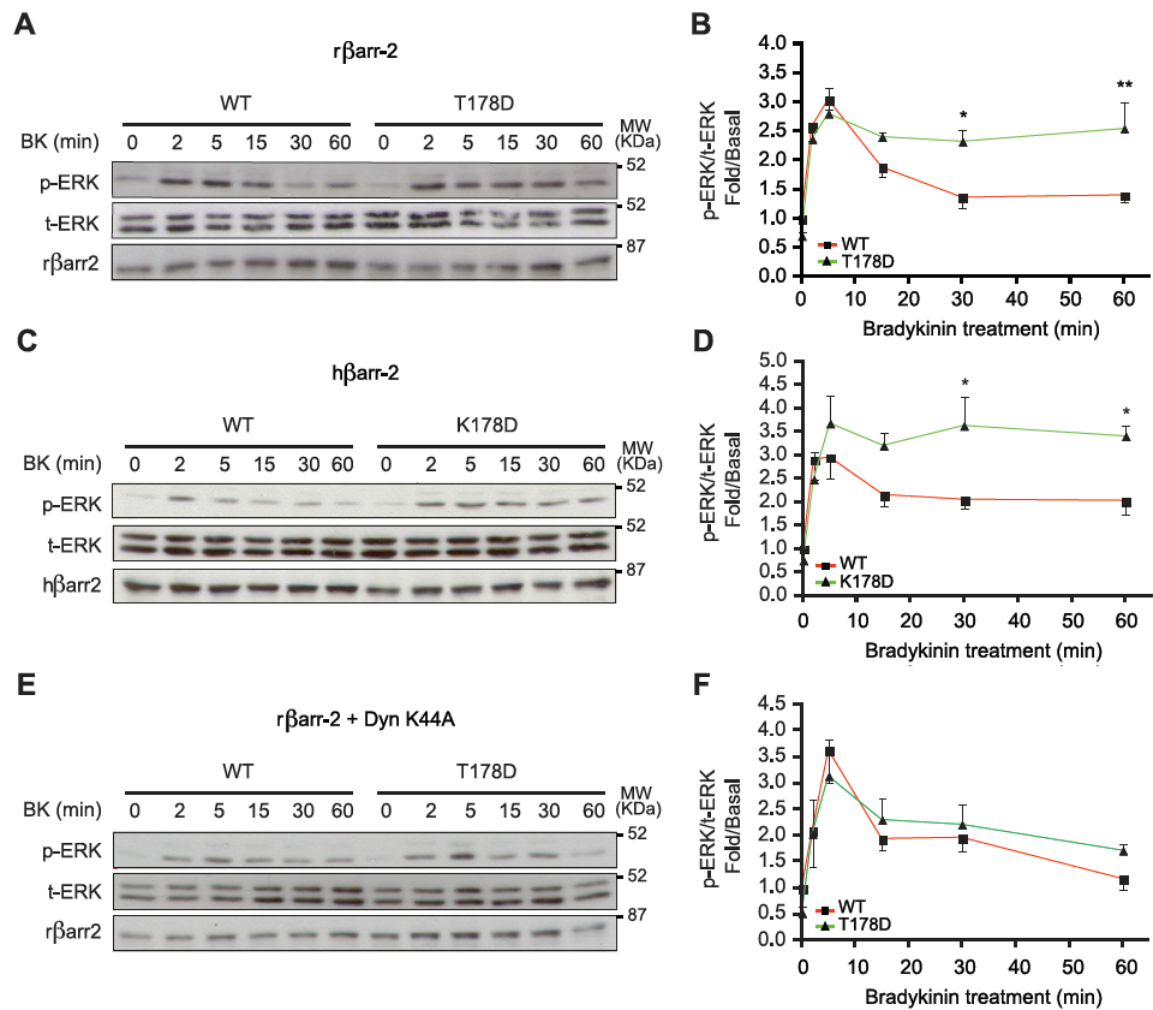


Figure 10. Aspartic acid substitution at position 178 of β -arrestin-2 promotes sustained B2R-dependent MAPK activation. (A, C, E) Shown are representative immuno-blots of ERK activation from COS-7 cells expressing HA-B2R and either (A) rat β -arrestin-2 (r β arr-2) WT or r β arr-2 T¹⁷⁸D mutant, and (C) either human β -arrestin-2 (h β arr-2) WT or h β arr-2 K¹⁷⁸D mutant, and challenged with bradykinin (1 μ M) for the indicated time. (E) Cells were transfected as in (A) with the addition of Dynamin K⁴⁴A (Dyn K⁴⁴A). Lysates were immunoblotted for phosphorylated ERK (p-ERK), total ERK (t-ERK) and β -arrestin-2 (BARR3978). Molecular markers of 52 and 87 KDa are depicted. (B, D, F) Densitometry analysis of data from A, C and E, respectively, which represents the mean $\pm$ SEM of minimum three independent experiments. p-ERK signals were normalized to that of t-ERK, and compared to non-treated cells for statistical analysis using a two-way ANOVA followed test followed by a Bonferroni post-test. *, $p < 0.05$, **, $p < 0.01$.

FIGURE 11

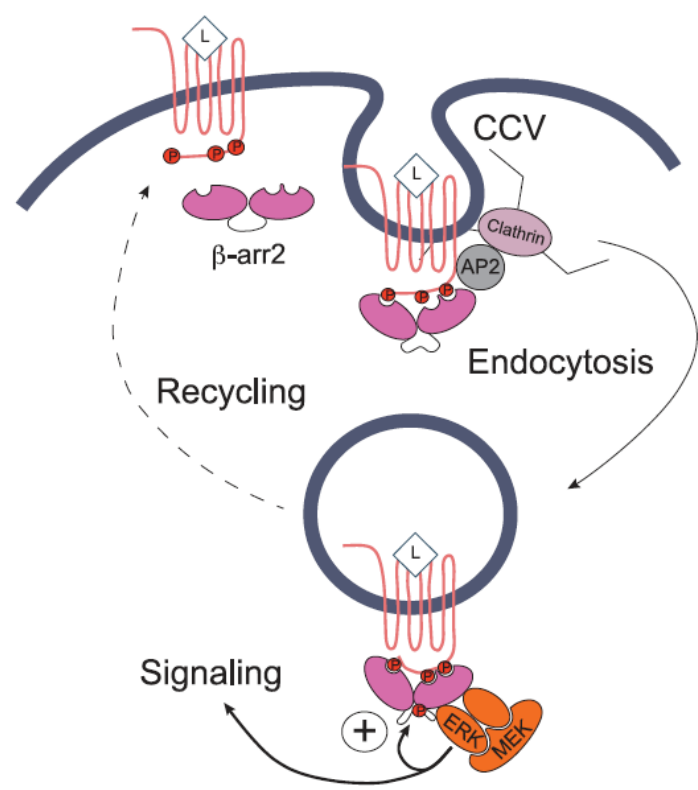


Figure 11. Positive feed back mechanism regulation of B2R trafficking and intracellular MAPK signaling by the scaffolding function of β -arrestin-2. Ligand binding to B2R promotes G protein-kinase-dependent receptor phosphorylation, the binding of β -arrestin-2 (β -arr2) to receptors and the clathrin-dependent internalization of the complex into endosomes. The B2R/ β -arrestin-2 then recruits a MAPK complex composed of MEK, ERK1/2 and presumably Raf (not shown) for intracellular signaling. The introduction of a negative charge in the hinge domain of β -arrestin-2, such as in the case of the phosphorylation of Thr¹⁷⁸ by MAPK, increases furthermore the avidity of the B2R/ β -arrestin-2 complex, allowing continued ERK1/2 signaling in the endosome, which further maintains, through a positive feed back mechanism, the high affinity binding state of the complex, leading to slower recycling of the receptor to the plasma membrane. CCV, clathrin-coated vesicles; AP2, clathrin adaptor protein 2.

Connecting text

Background: The trafficking and signaling functions of β -arrestins are studied through several approaches, including genetic tools, such as arrestins dominant negative mutants and knock-down of the arrestin expression in cells (siRNA). The lack of any pharmacological tool capable of modulating the interactions between β -arrestins and agonist-activated GPCRs was the main rationale of this study. The central aim of this project was to design a virtual screen based on the crystal structure of β -arrestin-1 and using the polar core of the arrestin as the target docking site. This approach will help us identify a selective pharmacological inhibitor of β -arrestin capable of blocking its interaction with the receptor.

Results: The virtual screen scored a hit rate of 6%, identifying a total of 4 active compounds amongst 66 compounds that were tested for receptor endocytosis. The results obtained from β -arrestin/receptor endosomal interactions, receptor recycling and arrestin cell signaling responses lead to the identification of one potent inhibitor of β -arrestin, namely UM0012685.

Conclusion: The virtual screening combined with cell-based assays reveals for the first time a selective pharmacological inhibitor of β -arrestin.

Significance: A first pharmacological tool to study several functions of β -arrestins on GPCR endocytosis, recycling and MAPK activation.

Chapter 4

β -arrestins Inhibitor Discovery by Combined Virtual Screening and Cell-based Assays

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Abstract

β arrestins (β arrs) are scaffolding proteins controlling G protein-coupled receptor (GPCR) endocytosis and signaling activities. Despite their importance, no selective pharmacological inhibitor of their function exists. We conducted a virtual screen based on the crystal structure of β arr1 and identified compounds that inhibit the recruitment of β arr1 and 2 to receptors and block the ensuing endocytosis. The most potent inhibitor, UM0012685, also inhibits the β arr-dependent activation of ERK1/2, affects the dynamic of β arrs-receptor association in the endosomes and revealed a role for this interaction in the recycling of receptors following endocytosis. The computationally predicted binding mode of UM0012685 to β arrs was confirmed by mutagenesis studies that suggest a competition between the compound and the phosphorylated carboxyl tail of the receptors for the polar-core region of β arrs. In addition to validate virtual screening to identify β arrs inhibitors, the study provides a pharmacological tool to study the diverse functions of β arrs.

Introduction

Arrestins form a family of accessory proteins that play a central role in regulating G protein-coupled receptors (GPCR) function. In mammals, 4 isoforms exist with arrestin1 and arrestin4 being mainly found in the photoreceptor cells of the visual system whereas arrestin2 and arrestin3, also known as β arrestin1 (β arr1) and β arrestin2 (β arr2), are ubiquitously expressed. As regulators of the largest class of membrane protein in the human genome that transduce signals from a wide variety of stimuli across biological membranes, they play a role in most physiological functions.

Originally discovered for their role in agonist-promoted desensitization and internalization of GPCRs following sustained activation, β arr1 and β arr2 were later found to have intrinsic signaling activities, namely through the scaffolding and activation of the mitogen-activated protein kinases (MAPK). The scaffolding role of β arrs for both endocytosis and MAPK activation requires their recruitment from the cytoplasm to the receptor, linking the receptor to components of the clathrin-mediated endocytosis machinery such as adaptor protein-2 (AP2) (1) and clathrin (2) as well as to signaling molecules like c-Src (3) and ERK1/2 (4). This process is greatly favored by the phosphorylation of the receptors by G protein-coupled receptor kinases (GRKs) that lead to an increased affinity of receptors for β arrs (5). Receptors having a scattered distribution of phosphorylated residues on their C-termini have weaker interaction with β arr and rapidly recycle back to the plasma membrane whereas those with clusters of phosphorylated residues have stronger interaction and remain for extended times in endosomes before being targeted to lysosome for degradation (6-8). These receptors are referred as class A and B, respectively.

Although β arrrs have been shown to play a role in the endocytosis of numerous GPCRs, this is not universal since some GPCRs have been shown to undergo β arrrs-independent endocytosis (9-15). For several of these receptors, however, β arrrs has been shown to be essential for the receptor recycling to the plasma membrane following endocytosis (9-11), thus suggesting a dual role of β arrrs in receptor trafficking that may differentially affect different receptors. The role of β arrrs in GPCR-stimulated MAPK activity has also been found to be highly variable from one receptor to another (4, 16, 17).

Since no selective pharmacological tool exists, all studies assessing the roles of β arrrs have relied on the use of genetic tools, including dominant negative mutants (10, 12, 18, 19), antisense- and siRNA- mediated knock-down (11, 20-22) and cells derived from β arrrs knock-out mice (9, 14, 15, 23-25). This lack of pharmacological tool has hampered our ability to assess, on a large scale and in various biological systems, the relative contribution of β arrrs to the various functions in which they have been implicated. Yet, the structural information gathered about β arrrs and their mode of activation offer opportunities for the development of small molecular modulators. The structural features of the two β arrrs are strictly conserved and include an unstructured C-terminal tail and two β -sandwich folds called the N- and C-domains that bind phosphorylated receptors through their concave polybasic surface. At the interface of the N-domain, C-domain and the C-termini resides a polar core formed by an interacting network of electrostatic residues important to keep the β arrrs in their inactive state (26, 27). The phosphorylated carboxyl-tail of GPCRs binds to the β arrrs N-domain, thus leading to the disruption of the polar core and the release of the core-embedded C-termini region that contains the

residues involved in the interaction with downstream effectors such as AP-2 (28-31). Compounds that could bind to the polar core region of β arrs would therefore be predicted to modulate their receptor-mediated activation, thus providing useful tool compound to study β arrs functions.

The aim of the present study was to identify small organic pharmacological modulators of β arr activity. For this purpose, a virtual screen was performed on a model derived from a β arr1 crystal structure using a grid that included the polar core region as the target. The activity of the virtual hits were then experimentally tested on both signaling and trafficking functions, leading to the identification of the first pharmacological inhibitor of β arrs. In addition to allow studying the role of β arrs in agonist promoted endocytosis and MAPK activation, this new tool compound allowed to investigate the dynamic of β arr-receptor interactions in the endosomes and its role in receptor recycling.

Experimental Procedures

Material - Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, glutamine and phosphate buffer saline (PBS) was from Wisent (ST-Jean-Baptiste, QC, Canada). Cell culture plates, dishes and costar 96 wells v-bottom polypropylene plates were from Corning (Tewksbury, MA). Bovine Serum Albumin (BSA), Hygromycin B, 8-Arginine-Vasopressin (AVP), Platelet Activating Factor (C₁₆), AngiotensinII and (-)-Isoproterenol were from Sigma (St-Louis, MO). Candidate compounds were from Sigma (St-Louis, MO) and Chembridge (San Diego, CA). Polyethylenimine (PEI) 25 kD for DNA transfection was from Polysciences, Inc (Warrington, PA). All DNA restriction enzymes are from New England Biolab (Ipswich, MA). Coelenterazine h was purchased from Prolume (Pinetop, AZ). The mouse monoclonal anti-HA.11 antibody (16B12) was from Covance (Princeton, NJ). The rat monoclonal anti-HA (3F10) horseradish peroxidase-conjugated antibody was from Roche (Mississauga, ON, Canada). The chicken anti-mouse IgG Alexa Fluor 647 antibody, Lipofectamine2000 and pcDNA3.1zeo(+) were from Invitrogen (Burlington, ON, Canada). All oligonucleotide were synthesized at Biocorp (Montréal, QC, Canada) except those for pIRESpuro3a vector that were from IDT (Coralville, IA). Polyornithine was from Sigma-Aldrich (St. Louis, MO). DCtm protein assay was from Biorad (Mississauga, ON, Canada). Optiplate and Culturplate microplates were from PerkinElmer (Wellesley, MA).

Plasmids and constructs - The expression vectors pIRESHygro3 and pIRESpuro3

were from Clontech (Mountain View, CA). The expression vector phRLuc-N2 was from Perkin-Elmer (Montréal, QC, Canada). The expression vector pcDNA3.1Zeo(+) was from InVitrogen (Burlington, ON, Canada). The expression vector pcDNA3.1(+)-3HA-V2R was from Missouri S&T cDNA resource center (Rolla, MO). The plasmids encoding HA- β 2AR46, β arr1-RlucII (40), B2R-YFP (47), HA-V2R (8), HA-B2R (47), B2R-V2CT (47), β arr2-YFP (47), V2R-venus (36), β arr2-RlucI (36) has been described previously. To create pIRESpuro3a vector, two complementary oligonucleotides (5'-aattcagggatccaggtgtacagactcgagtcgc-3' and 5'-ggccgcgactcgagtctgtacactggatccctg-3') containing alternative restriction sites were hybridized and inserted in pIRESpuro3 vector using EcoRI and NotI restriction enzymes. To make pIRES-HA-venus vector, the venus gene was PCR amplified and inserted into pIRES-HA vector described previously (48). To make HA-PAFR-venus encoding plasmid, *pafr* gene was PCR amplified and inserted in pIRES-HA-venus vector by recombination using In-Fusion PCR Cloning Kit from Clontech (Mountain View, CA). The HA-AT1R-venus encoding vector was made by replacing HA-PAFR gene in the HA-PAFR-venus encoding plasmid by an amplified HA-AT1R gene with a signal peptide (MKTIIALSYIFCLVFA) at the N-terminus. The plasmid encoding HA-V2R-venus was made in two cloning step. First, HA-V2R gene was PCR amplified from pcDNA3.1(+)-3HA-V2R plasmid, keeping one HA tag and removing the stop codon. The PCR product was then inserted in pIRESpuro3a vector. Then, the *venus* gene was PCR amplified from phRLuc-venus vector previously described (36) and inserted in pIRESpuro3a-HA-V2R. The resulting *HA-V2R-venus* fusion gene was then inserted into pIREShygro3 vector, yielding pIREShygro3-HAV2R-venus plasmid. To make 6his- β 2AR-YFP encoding vector, the *his- β 2AR-eYFP* fusion

sequence, consisting of a 6 x histidine tag, the human β 2AR sequence, a linker and the *eYFP* sequences, was inserted into pcDNA3.1zeo(+) vector. To make β arr2-hRLucII encoding vector, the coding sequence of human β arr2 gene was amplified by PCR and inserted in the previously described pcDNA3.1-GFP10-EPAC-hRLucII vector (49), removing the *gfp10-epac* gene in the process. To make the β arr2 mutants (R170E, Q131A, F391A, D68A, R393A), two pairs of oligonucleotides containing the mutation were used to amplify the wild type β arr2 gene. The two overlapping fragments were passed through a second round of amplification using the two oligonucleotides hybridizing at both extremities of the β arr2 gene. The mutated amplified β arr2 genes were then inserted in pcDNA3.1 β arr2-hRLucII, replacing the WT- β arr2 sequence. All clones were verified by sequencing.

Transfection, cell culture and stable cell line generation - HEK293T cells were cultured in DMEM supplemented with 10% FBS, 2mM glutamine, 0.1 unit/ml penicillin, 0.1 mg/ml streptomycin in a 5% CO₂ atmosphere at 37⁰C. Cells were transfected using a PEI transfection procedure. Briefly, PEI and DNA were mixed at a 3:1 mass ratio in DMEM, incubated for 15 min at room temperature and added to HEK293 cells. Two μ g of DNA in 200 μ l of DMEM and 10 μ g of DNA in 1 ml of DMEM was used for 6 wells culture plate and 100 mm petri dishes, respectively. Culture medium was changed after 2 hours. Experimental procedures were performed 24 to 48 hours after transfection. Generation of the HEK293 cells stably expressing the fusions protein V2R-venus and β arr2-RLuc has been previously described (36). HEK293 cells stably expressing hRLuc and HA-V2R-venus proteins were created by transfecting phRLuc-N2 or HA-V2R-venus

encoding vector using lipofectamin 2000 according to manufacturer's recommendation. Twenty-four hours post-transfection, cells were seeded 1/100 in 100 mm petri dishes. Cells were then selected using G418 (400 µg/ml, pHRLuc-N2) or hygromycin B (100 µg/ml, pIRESHygro3HA-V2R-venus). After one month of selection, polyclonal population were frozen.

Virtual screening and molecular docking - ChemAxontm and Openbabel softwares were used to derive 3D coordinates from compound librairies. MGLTools 1.5 software was used to prepare compounds for docking energy evaluation by Autodock4 software. Side chains or backbone of residues R62, S126, T128, Q130, V142, D143, E145, L166, V167, I168, R169, L289, D290, G291, K292, H295, D297, T298, F391, A392, R393 are forming the docking surface on β arr model. For each compound, 30 (Fig. 1) rounds of energy evaluation were performed using genetic/local search hybrid algorithm and similar conformations (RMSD < 2Å) were regrouped. The docking results were then analyzed using MGLTools-1.5 software. The conformation having the best affinity within the largest group (Fig. 1) was retained to sort compounds by order of decreased affinity. Structural homologues (95% structural identities) of the selected candidates in the IRIC library were selected using ChemAxontm software. For the docking of UM0012685 enantiomers (Fig. 3), 100 rounds of energy evaluation were performed, the conformation with the best overall affinity (Fig. 3) being retained for further analysis. Energy evaluation and data analysis has been automated using bash shell scripting. Two-dimensional compounds representations were generated with ChemBioDrawtm Software (CambridgeSoft). Three-dimensional molecular

representations were generated using Pymol software (PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

ELISA assay - HEK293 cells expressing HA-tagged receptors or HA-V2R-venus (Fig. 1a) were resuspended and plated at a density of 50,000 cells per well in a 96 wells white Culturplate microplates pre-treated with poly-ornithine. Following an additional 24 hours, cells were washed twice with PBS, subjected to the indicated pharmacological treatment at room temperature (Fig. 2a) or 37⁰C (Fig. 7b), fixed with 3% paraformaldehyde and rinsed three times with PBS. Non-specific antigen sites were blocked with PBS containing 0.5% BSA prior to the labeling of the HA tag on the cell surface using an horseradish (HRP)-conjugated anti-HA antibody for 1 hour. Excess antibody was removed by rinsing the cells three times with PBS. HRP chemiluminescence reactions were monitored shortly after the injection of Western Lighthouse Plus ECL, an HRP substrate, using a Mithras LB940 microplate reader without any emission filter. Receptor cell expression was normalized using GraphPad software, the receptor-expressing cells and mock exposed to vehicle being set as 100% and 0%, respectively.

FACS assay - HEK293 cells stably expressing the human type-2 vasopressin receptor (V2R) tagged at its N-terminus with a haemagglutinin (HA) epitope and fused to a yellow variant of the green fluorescent protein (venus) at the C-terminus (HA-V2R-venus) were washed twice and resuspended in HEPES tyrode's Buffer (140mM NaCl, 2,7mM KCl, 1mM CaCl₂, 12mM NaHCO₃, 5,6mM D-glucose, 0,49mM MgCl₂, 0,37mM

NaH₂PO₄, 25mM HEPES pH 7.4). Four hundred thousand cells, dispensed per well in a 96 wells v-bottom polypropylene plate, were subjected to the indicated pharmacological treatment at room temperature. Cells were then kept at 4°C to prevent further trafficking events, pellet by short centrifugation and buffer medium was removed. Cells were then shortly incubated in HEPES Tyrode's buffer supplemented with 1% BSA to block non-specific interaction followed by 30 min incubation with anti-HA mouse monoclonal antibody in the same buffer. Cells were then washed twice with HEPES Tyrode's buffer/1% BSA to remove excess of the primary antibody and incubated with anti-mouse Alexa Fluor 647 fluorescent antibody for 30 min. Cells were then washed twice with HEPES Tyrode's buffer / 1% BSA and once with HEPES Tyrode's buffer prior to being resuspended in HEPES Tyrode's buffer. Cells were analyzed using a LSR II flow cytometer (BD Biosciences, San Jose, CA) set to detect in distinct channels. Venus fluorescent protein was excited with a sapphire blue laser (20 mW, 488 nm) and detected through longpass 505 ± 15 nm followed by 530 ± 15 nm emission filters. Alexa Fluor 647 was excited with a HeNe laser (18 mW, 633 nm) and detected through a 660 ± 10 nm emission filter. Percentage of AVP-induced V2R endocytosis was determined as follow: HA vs venus signals representing the surface and total receptor expression, respectively, were plotted as X and Y axis on a two dimensional graph. Vehicle- and agonist-treated samples were compared and a polygonal gate was drawn around the dynamic population. Percentage of AVP-mediated endocytosis was determined using graphpad software normalization function by setting the 0% of endocytosis as the fraction of total cell in the gate after vehicle stimulation and 100 % as the fraction of total cell in the gate after AVP stimulation. Data analysis was performed using BD FACSDiva software.

BRET on stable cell line - One hundred thousand HEK293 cells expressing V2R-venus and β arr2 fused to Renilla luciferase (RLuc) at its C-terminus (β arr2-RLuc) and resuspended in HEPES tyrode's Buffer were dispensed per well of a 96-wells white Optiplate microplates from PerkinElmer (Wellesley, MA). Following the indicated pharmacological treatment at room temperature (RT), 5 μ M final concentration of coelenterazine-*h* was added to the cells suspension and luminescence signals were collected using a Mithras LB940 microplate reader from Berthold Technology (Bad Wildbad, Germany). BRET ratio is defined as the signal detected through energy acceptor filter (530 ± 20 nm) over the one detected through energy donor emission filter (480 ± 20 nm). The net BRET is the BRET ratio in the presence of the energy donor and acceptor pair minus the BRET ratio obtained only in the presence of an energy donor (see Supplementary Fig. 1).

BRET on transiently transfected cells - Twenty-four hours after transfection, HEK293 cells expressing BRET pair were resuspended and plated at a density of 50,000 cells per well in a 96 wells white Culturplate microplates pre-treated with poly-ornithine and then cultured for an additional 24 hours. The attached cells were then washed twice with HEPES tyrode's buffer and subjected to the indicated pharmacological stimulations at 37°C prior to coelenterazine-*h* addition and luminescence signal reading, as described above. Ligand-induced BRET is the BRET ratio from ligand-treated cells minus BRET ratio from vehicle-treated cells.

Western blotting - ERK1/2 was monitored as previously described (20). Pharmacological stimulations were performed at 37°C. Only 25 % of the total FBS-stimulated samples were loaded on the gel. Specific bands were detected using a LAS-3000 CCD camera device from Fujifilm and their densitometric analysis performed using Multi Gauge software from Fujifilm and ImageJ.

Confocal microscopy - HEK293 cells were plated in 35 mm glass bottom dishes and transfected with the indicated receptor and β arr2-YFP. Forty-eight hours post-transfection, cells were serum starved, stimulated with the indicated agonists to induced endosome formation followed by a stimulation with the indicated compounds prior to imaging of β arr2-YFP fluorescence. Two thousand forty-eight by 2048 pixels images were acquired at the indicated time. For fluorescence recovery after photobleaching (FRAP), selected endosomes were bleached and fluorescence recovery was monitored over a period of 3 min with images acquisition every 30 seconds. The region of interest was bleached for 100 iterations using 514nm laser set at 100% intensity. Images were collected on a Zeiss LSM-510 Meta laser scanning microscope with a 60x oil immersion lens using an Argon 2 laser with single line excitation at 514nm, and emission BP 530-600 nm filter sets for YFP. Fluorescence intensity data are expressed as percentage recovery in function of time (second) using GraphPad software (Prism) and converted into a linear regression plot to obtain maximal recovery and half-time recovery of the endosomal β arr2 / receptor complex, as described previously (40, 41). For quantification, endosomes were selected as round hollows displaying a diameter of less than 1 μ m and their density was determined using ImageJ software.

Statistical analysis - Statistical analysis and curve regression were carried out using GraphPad Prism 4 software (GraphPad Software Inc.).

Results

Virtual screening strategy

A major site of interaction between GPCRs and β arr for which known structural elements could guide the selection of the binding site of small organic modulators is the central electrostatic network at the interface of the N and C domains known as the polar core (Fig. 1a). For rhodopsin, type 1 cannabinoid receptor and type-2 vasopressin receptor (V2R), this region was reported to bind directly to the phosphorylated carboxyl tail of the receptor (29, 30, 32, 33). Therefore, the strategy selected to identify β arr modulators was to search for small molecules targeting the surface of the polar core of β arr using virtual screening. However, crystal structures show that the polar core is partially hidden by the ‘finger loop’ (residues 64-74 for β arr1), creating a lid that precludes direct access to its surface (26, 34). To allow access of small molecules to the surface of the polar core region, we used a β arr1 model derived from its crystal structure (1ZSH) in which loop 64-74 has been displaced out of the polar core entry (Fig. 1a, lower panel). The exposed solvent-accessible surface is conserved among β arr subtypes and species (Fig. 1b) and forms a pocket of 636 Å² (2), including the polar core region, that was used to evaluate the binding energies of 30,000 compounds from three commercially available libraries (Myriascreen, Actiprobe10 and TimTec diversity sets from Sigma) (Fig.1c). These libraries are structurally representative of a much larger collections of 2,500,000 drug-like compounds. These compounds were fitted in a docking grid using the genetic algorithm of the Autodock software (see methods). Ranking the 30,000 compounds according to their docking energy, 517 were found to have a predicted

binding affinity of less than 2 μ M. In addition to these candidates, 91 closely related structural homologues were selected from 79,648 chemicals of the IRIC chemical library (<http://www.irc.ca/recherche/infrastructures/criblage-a-hautdebit/?section=technologies>), providing a total pool of 608 candidates. Among this pool, 66 candidates were acquired based on their availability and affordability and were further characterized (Fig. 1c).

Validation of candidates

To assess the potential action of these compounds on β arr functions, we first tested their effect on agonist-promoted GPCR endocytosis. Figure 2a illustrates that stimulation of the human type-2 vasopressin receptor (V2R) stably expressed in HEK293 cells with its natural agonist, arginine vasopressin (AVP) for 20 minutes led to significant endocytosis resulting in a loss of 59 % of the cell surface V2R as assessed by ELISA. To test the effect of the 66 compounds, we used a dual flow cytometry (FACS) assay that simultaneously detects the total and cell surface V2R expression, reflected by the intrinsic fluorescence of a C-terminal yellow variant of green fluorescent protein (venus) tag and immunofluorescence of a N-terminal HA tag, respectively. Such quantification revealed that 8 out of the 66 tested compounds significantly inhibited AVP-stimulated endocytosis when added to the cell at a concentration of 100 μ M, 10 minutes prior to AVP stimulation (Fig. 2b). The inhibiting activity of the 4 most efficacious compounds (23, 38, 52 and 57) was confirmed in dose-dependent curves (Fig. 2c), yielding inhibition of more than 50% of the endocytosis at maximal concentration. IC₅₀s could be calculated for 3 of the compounds (23, 38, 57) with potencies between 6 and 32 μ M. None of these compounds had effect on receptor cell surface expression in the absence of AVP

stimulation (Supplementary Results and Supplementary Fig. 1), indicating that the compounds selectively block agonist-promoted endocytosis.

To confirm the mechanism of action of the inhibitory compounds, we used a BRET-based assay that directly monitors the interaction between β arrs and GPCRs (35, 36). Six out of the 8 endocytosis inhibitors were spectrally active and interfered with BRET (Supplementary Fig. 2), thus only compounds 37 and 57 were further characterized.

Stimulation of cells with a saturating concentration of AVP promoted a significant increase in BRET (Fig. 2d), reflecting recruitment of β arr2-RLuc to the V2R-venus. Both compounds 37 and 57 significantly inhibited the AVP-stimulated BRET (Fig. 2d, left panel), indicating that they prevented the recruitment of β arr2 to the receptor. In the absence of AVP stimulation neither compound had an effect on the BRET signal (Fig. 2d, right panel). Two compounds (6 and 18) that did not affect AVP-stimulated endocytosis were used as negative control. They were without effect on the agonist-mediated recruitment to V2R, confirming the selectivity of the assay. A dose-response curve of the most potent endocytosis inhibitor (compound 57) revealed a $\log IC_{50}$ of -5.5 ± 0.2 for the inhibition of β arr2 recruitment to the V2R (Fig. 2e), a value almost identical to that observed for the inhibition of endocytosis (-5.2 ± 0.2 ; Fig. 2c, lower right panel), consistent with a common mode of action of the compound for the two responses. Compound 57 also blocked the recruitment of β arr1-RLuc to V2R-venus with a comparable potency ($\log IC_{50} -5.4 \pm 0.2$), indicating that the compound is an inhibitor of both β arr1 and β arr2 recruitment as expected from their highly conserved predicted binding surface (Fig. 1b). Given that the docking grid used for the virtual

screen targeted one of the major binding sites of β arr to the receptor, it can be proposed that compound 57 acts by directly interfering with the association of β arr to the V2R. Consistent with this notion, compound 57 does not act as a competitive antagonist of the V2R as it did not affect [3 H]-AVP binding to the V2R (Supplementary Fig. 3). Thus, compound 57 was selected for further analysis and will now be referred to as compound UM0012685.

Predicted binding mode of UM0012685

Since UM0012685 harbors a chiral center on carbon 4 of the quinoline core moiety (Fig. 3a), both enantiomers were docked on the β arr model. The "S" enantiomer showed a 1.1 kcal / mol lower predicted binding energy than the "R" enantiomer and was thus used for binding mode analysis (Fig. 3). Figure 3b illustrates the pose of UM0012685 in the β arr1 binding pocket. Seven residues from the polar core region were predicted to directly contact UM0012685. The cyclopentyl group forms hydrophobic interactions with the side chains of residues Ser126, Thr128 and Val167 that, together, form a cavity at the bottom of the binding pocket. The bromophenyl group interacts with the guanidinium group of Arg62 through the bromine atom whereas the side chain of Val167 coordinates the bromophenyl aromatic moiety. The nitrogens from Lys292 backbone and His295 side chain engage in electrostatic bonds with the carbonyl of the UM0012685 ester function. Similarly, the carbonyl of the Val167 backbone and the guanidinium of the Arg393 side chain interact with the nitrogen of the compound quinoline core. Phe391 also forms hydrophobic contacts with the quinoline core on carbons 7 and 8.

To test the contribution of the predicted binding pocket on UM0012685 efficacy, the role of Phe391 and Arg393, which contribute to the formation of the polar core structure by anchoring the carboxyl terminal of β arr in the N-domain, was assessed by substitutions to alanine (F391A- β arr2; R393A- β arr2). As shown in Figure 3c, whereas UM0012685 inhibited the AVP-promoted recruitment of wild-type β arr2 to the V2R by 78%, the inhibitory action of the compound was significantly reduced to 46% and 45% for F391A- β arr2 and R393A- β arr2, respectively, confirming the importance of these residues for the action of UM0012685. Mutations of two other residues in the docking surface of β arr2 (D68A and Q131A), which are not predicted to interact with UM0012685, did not affect its inhibitory action, again supporting the binding mode proposed by the docking experiments. The fact that mutations Arg393 and Gln131 promoted greater AVP-stimulated β arr2 recruitment is not surprising given the role of this region in the interaction with the receptor. However, their effect on the inhibitory action of UM0012685 was independent of such elevation since Q131A, which caused the greatest enhancement of the AVP-stimulated BRET, did not affect the inhibitory activity of UM0012685.

Also consistent with the binding mode proposed by the virtual docking are the results obtained with analogues of UM0012685 (Fig. 3d) where the bromine atom in the 'para' position of the phenyl group was substituted by either a hydrogen (compound 57a) or a methyl ester group (compound 57b). Whereas the hydrogen substitution, which cannot mimic the electrostatic halogen bonding with Arg62, abolished the inhibitory activity of the compound, the replacement with the methyl ester hydrogen bond acceptor preserved significant inhibitory activity (Fig. 3d). Taken together, these results indicate

that UM0012685 acts through its binding to the polar core region of β arr2 thus most likely blocking access to the receptor carboxyl tail.

UM0012685 selectivity of action

To assess whether UM0012685 could inhibit the recruitment of β arr to other GPCR family members, UM0012685 was tested against agonist-stimulated recruitment of β arr2 to β 2-adrenergic receptor (β 2AR), platelet activation factor receptor (PAFR), type 1 angiotensin II receptor (AT1R) for which phosphorylation of their carboxyl tail has been shown to be important for the recruitment of β arr2 (6-8, 37). As observed for the V2R, UM0012685 significantly blocked the agonist-promoted recruitment of β arr2 to these receptors (Fig. 4). However, it was without effect on the bradykinin-stimulated recruitment of β arr2 to the type-2 bradykinin receptor (B2R), which has been suggested to be independent of the receptor's carboxyl tail phosphorylation (38), consistent with the proposed action mode of UM0012685. Indeed, since the phosphorylated carboxyl tail is believed to play its role by destabilizing the polar core interactions, resulting in the release of the C-termini from the N-domain and the opening of this region, the difference in the recruitment mode of β arr2 by the B2R most likely explains the lack of action of UM0012685 on the β arr2/B2R interaction. To test this hypothesis, we took advantage of a pre-activated mutant form of β arr2 that can interact with high affinity to non-phosphorylated receptors, β arr2 R170E (39), presumably as a result of a spontaneous opening of the polar core region. As shown in Figure 4b, contrary to what was observed for the wild type β arr2 (Fig. 4a, lower right panel), the bradykinin-stimulated recruitment of β arr2 R170E was significantly blocked by UM0012685. Mutation of another residue

located in the docking surface of β arr2, β arr2 Q131A, did not have the same effect than the R170E activating mutation, confirming the selectivity of the effect. For V2R, UM0012685 similarly inhibited the AVP-promoted recruitment of both wild-type β arr2 (Fig. 2d) and β arr2 R170E (Fig. 4b) to the V2R indicating that the effect of the Arg170 mutation on the inhibitory activity of UM0012685 is specific to the mode of engagement of β arr2 by the receptor. These data are consistent with a model whereby UM0012685 requires the opening of the polar core region of β arr2 to be active (Supplementary Fig. 5).

Probing β arr functions with UM0012685

To assess the usefulness of UM0012685 as a chemical tool to study different β arr functions, we first assessed the effect of the compound on a well-characterized signaling activity of β arr, the V2R-stimulated ERK1/2 activation (17, 20). As shown in Figure 5a, UM0012685 significantly blocked the AVP-stimulated ERK1/2 activation while not significantly affecting the FBS-stimulated activity, validating the use of UM0012685 to probe receptor-stimulated β arr functions.

Previous studies have suggested that stable interaction between GPCRs and β arr regulates the recycling of the receptor. In particular, it has been proposed that the lack of recycling of some GPCRs, known as class B receptors (6), may result from their prolonged interaction with β arr in the endosomes. To test whether blocking β arr/receptor interaction could affect the recycling of either class B or rapidly recycling class A receptors, we assessed the effect of UM0012685 on the recycling pattern of V2R (class B) and β 2AR (class A). Recycling was assessed by monitoring the reappearance of receptors at the cell surface by ELISA after a 30 minutes treatment with agonists (to

induce endocytosis) followed by the removal of the agonist and a recycling period of 60 minutes in the presence or absence of UM0012685. For both β 2AR and V2R, stimulation with agonists led to a $\sim$ 30% reduction in cell surface receptor density, reflecting endocytosis (Fig. 5b). As expected for class B receptors, no reappearance of cell surface V2R was observed 60 minutes following the removal of the agonist whether in the presence or absence of UM0012685. In fact, the addition of UM0012685 led to further decrease in cell surface V2R, suggesting that it blocked the little recycling that could occur. For β 2AR, as expected for a class A receptor, 66% of the lost-cell surface receptor had reappeared 60 minutes after agonist removal. In the presence of UM0012685, this recycling was completely blocked. Taken together, these results indicate that UM0012685 treatment following endocytosis had little effect on the recycling pattern of V2R but largely impaired the recycling of the β 2AR, indicating that receptor/ β arr interaction may play an important role in class A receptor recycling.

The observation that UM0012685 did not favor the recycling of the V2R may appear paradoxical given the role proposed for the endosomal β arr/receptor interaction in restricting recycling. To directly test whether UM0012685 could directly influence the interaction between β arr2 and V2R in the endosome, we took advantage of recent data demonstrating dynamic association-dissociation cycles that can be observed using recovery after photobleaching (40). As shown in Figure 6a, V2R activation upon AVP treatment led, as previously reported, to the accumulation of the V2R-bound β arr2-YFP in endosomes that appear as small circular discrete hollows in the cytoplasm (8). Treating the cells with UM0012685 for 30 minutes, in the presence of the agonist, led to a significant reduction of the number of β arr-positive endosomes, consistent with

UM0012685 inhibiting the association between V2R and β arr2 in the endosomes. Fluorescence recovery after photobleaching (FRAP) was then used to monitor the lifetime of the β arr2/V2R complex in endosomes (40-42). As shown in Figure 6b-d UM0012685 led to a 47% reduction in the half-time of recovery after photobleaching (from 47.07s to 24.93s) (Figure 6d). A closely related compound that does not block the interaction between β arr2 and V2R did not affect the recovery after photobleaching (Supplementary Fig. 4), confirming the selectivity of action of UM0012685. These data are consistent with the idea that UM0012685 inhibits the interaction between V2R and β arr2 in the endosome resulting in an increased rate of exchange between the cytoplasmic fluorescent β arr2-YFP and the bleached endosomal-bound β arr2. Consistent with our observation that UM0012685 did not inhibit the BRET between B2R-YFP and β arr2-Rluc2, the half-time for the recovery after photobleaching for the endosomal B2R/ β arr2 complex was not significantly affected by the treatment with the compound (Fig. 6d). However, using a mutant form of the B2R harbouring the carboxyl tail of the V2R (B2R-V2RCT), UM0012685 significantly reduced the half-time of recovery after photobleaching for the endosomal β arr2 indicating that the mode of engagement of β arr by the phosphorylated tail of the receptor plays a critical role for the action of UM0012685 in endosomes.

Discussion

The combination of a virtual screening based on a model derived from a crystal structure with cell based assays lead to the identification of the first β arr inhibitor thus providing a pharmacological tool to directly study the functions of β arrs upon GPCR activation.

The target grid used for the virtual screening was based on the crystal structure of bovine β arr1 in a basal inactive state. Because we wanted to identify compounds that would interfere with the binding of the phosphorylated receptor's carboxyl tail, we modified the position of the loop 64-74 (also known as the finger loop) to allow access of compounds to the polar core domain that is disrupted upon binding of the phosphorylated carboxyl tail of the receptor. In retrospect, this artificial modification of the structure turned out to be very similar to the position of the finger loop in the recently determined structure of both β arr1 and p44-arrestin in the active conformation (30, 43).

The virtual screen resulted in a pre-selection of virtual hits that allowed identifying 4 active compounds out of the 66 experimentally tested for a hit rate of 6%. This compares advantageously to the usual hit rate of 0.1% typically observed for traditional high throughput screens, thus validating the virtual screening approach and the model used. The virtual docking also allowed predicting a binding mode for the most active inhibitor, UM0012685, to β arr. This predicted binding site involves residues that either interact directly with the receptors' carboxyl tail (R62) or that are within β arr domains (C-tail and lariat loop) that are heavily displaced upon receptors' C-tail binding to β arr (F391, R393, H295, K292). Through binding to these residues, UM0012685 most

likely stabilizes inactive basal states of β arr by competing with the receptor's C-tail for its interaction with R62 and by maintaining the C-tail of β arr in close apposition to its N-domain, thus creating steric hindrance for the binding of the receptor's C-tail (Supplementary Fig. 5). Moreover, stabilizing the lariat loop in inactive conformation precludes the correct positioning of the loop residues for receptor phosphates coordination, further decreasing receptor's C-tail binding. Although the relatively low solubility of the compound prevented the direct biophysical analysis of its binding site, the docking prediction is strongly supported by the observation that mutation of F391 and R393 greatly inhibited the action of UM0012685. The use of UM0012685 analogues also validated the interaction model (Fig. 3d).

The proposed binding mode in the polar core region is consistent with the observation that UM0012685 acts preferentially on the activated state of β arr following the opening of the finger loop promoted either by the binding of the receptor's carboxyl tail or the constitutive activation of β arr. This is supported by the observation that, whereas it does not inhibit the interaction between the WT- β arr2 and B2R, a receptor that does not rely on the phosphorylation of its C-tail (38), UM0012685 significantly blocked the recruitment of a constitutively activated form of β arr2 (R170E) to the B2R. The role of the C-tail of the receptors in the activity of UM0012685 is also supported by the fact that, whereas the compound did not affect the dynamics between the B2R and β arr in the endosome, it significantly affected the endosomal β arr-receptor dynamics for the V2R and for chimeric form of the B2R harbouring the V2R C-tail.

As expected, UM0012685 blocked agonist-promoted endocytosis of V2R with the same potency that it blocked β arr recruitment to the receptor, confirming its usefulness as

a tool compound to study the canonical role of β arr in receptor endocytosis. It also blocked the well characterized β arr-dependent V2R-promoted ERK1/2 activation, confirming that it can also serve as an inhibitor of signaling downstream of β arrestin activation. Although not universal since it could not blocked β arr recruitment to the B2R, UM0012685 blocked β arr recruitment to several GPCRs, a property that should be expected from a direct inhibitor of β arr preventing its recruitment to GPCRs, making it a good tool to study the role of β arr activation by many distinct receptors.

In addition to inhibit the canonical action of β arr on endocytosis and ERK1/2 activation, UM0012685 was also found to block β 2AR recycling. Although such action of β arr had never been shown for the β 2AR, it had been proposed for receptors such as the N-formyl peptide (9), the glucagon (10) and the CXCR7 chemokine (11) receptors. In fact, the absence, until now, of pharmacological inhibitors for β arr prevented to study the role of β arrs in the recycling of receptors that are endocytosed in a β arr-dependent manner. Indeed β arr gene inactivation or knockdown lead to inhibition of receptor endocytosis, making it impossible to study recycling. Our study shows for the first time that β arr can have a dual role and be important for both endocytosis and recycling of the same receptor. Although the mechanism by which β arr promotes recycling is unknown, it should be noted that β arr has been shown to interact with the ArfGAP, AGAP2, a protein involved in β 2AR recycling (44). UM0012685 should prove a useful tool to further study the role of β arr in β 2AR recycling and possibly other GPCRs.

For the V2R, a class B receptor that does not undergo recycling and remains associated for extended period with β arr in the endosome, UM0012685 inhibited such association in the endosome. Although it was previously suggested that the lack of

recycling of class B receptors results from the extended interaction with β arr (6), UM0012685 did not restore V2R recycling. This suggests that the stable interaction with β arr may not be the principal determinant of receptor retention in the endosomes.

In conclusion, our study validates the combined use of virtual screening and cell based assays to identify small molecules interfering with the engagement of the polar core domain of β arrs by GPCRs and provides a novel tool compound to probe the different roles of β arrs in receptor trafficking and signaling.

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Authors Contributions - M.A. helped in constructing the calculation platform, did the virtual screening, designed and performed all the bench experiments except the microscopy and wrote the manuscript. E.K. performed the microscopy experiment and participated in writing the manuscript. S.A.L. supervised the microscopy experiment and participated in writing the manuscript. W.S. constructed the β arr2RLucII encoding vector. V.L. constructed the HA-AT1R encoding vector. M.B participated in the writing of the manuscript and supervised the experiments. C.L. built the calculation platform and helped in designing and supervising the experiment. A.B. helped for the compound mining in the in-house library database.

Figures and Legends

FIGURE 1

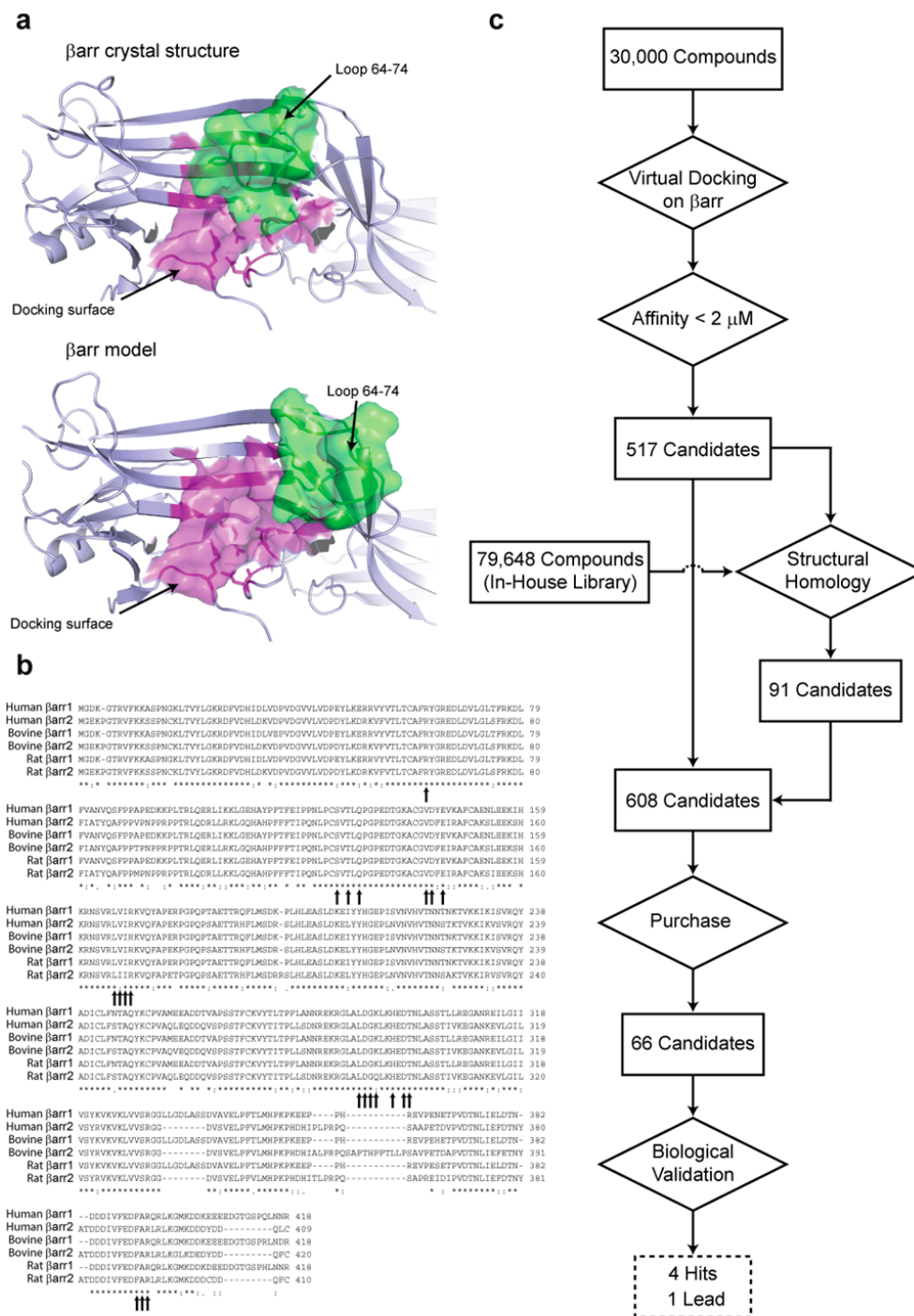


Figure 1. Virtual screening strategy. (a) Ribbon representation of β arr1 crystal structure (PDBID: 1zsh, upper panel) and the β arr model used for the virtual screening (lower panel). Transparent surfaces overlaid are the docking area (magenta) and loop 64-74 (green). Residues R169, D290, D297 and R393 from the polar core structural motif are represented as magenta sticks. (b) Multiple alignments of β arr1 and β arr2 protein sequences from different species using T-coffee algorithm (45). Sequences are from genbank (human β arr1: AAH03636.1, human β arr2: AAH67368.1, bovine β arr1: NP_776668.1, bovine β arr2: NP_001192206.1, rat β arr1: AAA74459.1, rat β arr2: AAH87578.1). Black arrows indicate residues located in the docking area. (c) Flowchart of the virtual screening approach.

FIGURE 2

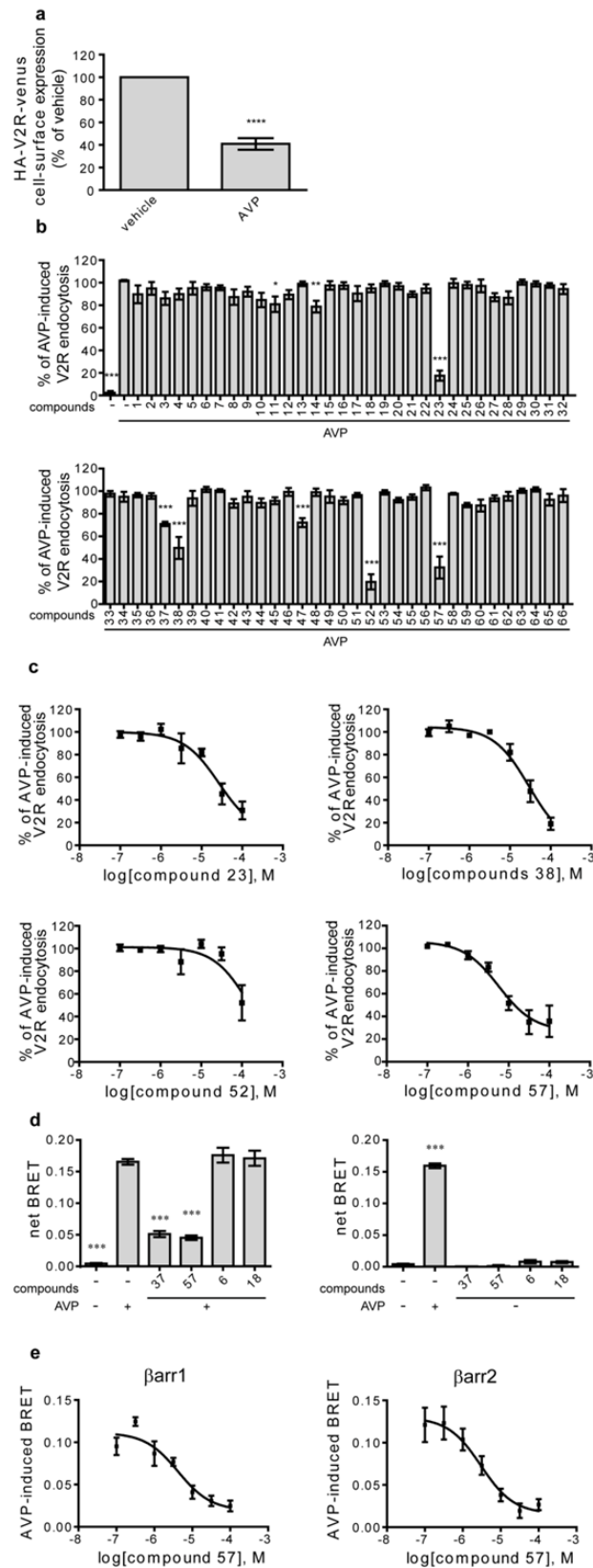


Figure 2. Validation of the virtual screen candidates. AVP-mediated V2R endocytosis in cells stably expressing HA-V2R-venus detected by (a) ELISA and (b,c) FACS. Cells were pre-treated with vehicle or candidates (100 μ M (b) or as indicated (c), 10 min), then stimulated with vehicle or AVP (100 nM, 20 min). logIC₅₀ $\pm$ s.e.m. are -4.6 ± 0.2 (compound 23), -4.5 ± 0.1 (compound 38) and -5.2 ± 0.2 (compound 57). (d-e) AVP-mediated β arrrs interaction with V2R detected by BRET. (d) Cells stably expressing V2R-venus and β arr2-RLuc were pretreated with vehicle or candidates (100 μ M, 10 min) and then treated with vehicle or AVP (100 nM, 40 min). (e) Cells transiently expressing V2R-eYFP with β arr1-RLucII or β arr2-RLucII were pre-treated (25 min) with increased concentration of compound 57 and then treated with AVP (100 nM, 5 min). logIC₅₀ $\pm$ s.e.m. are -5.4 ± 0.2 (β arr1) and -5.5 ± 0.2 (β arr2). Data represent the mean $\pm$ s.e.m of 3 to 8 independent experiments. Significance against 100% cell surface receptor expression (ELISA assay) was determined using onesample t test (****P < 0.0001). Significance against vehicle pre-treated/AVP-treated sample in the FACS assay was determined using ANOVA followed by Tukey's test. (***P < 0.001; **P < 0.01; *P < 0.05). Significance against vehicle ((d), right panel) or vehicle pre-treated/AVP-treated ((d), left panel) sample was determined using ANOVA followed by Dunnett's test (***P < 0.001).

FIGURE 3

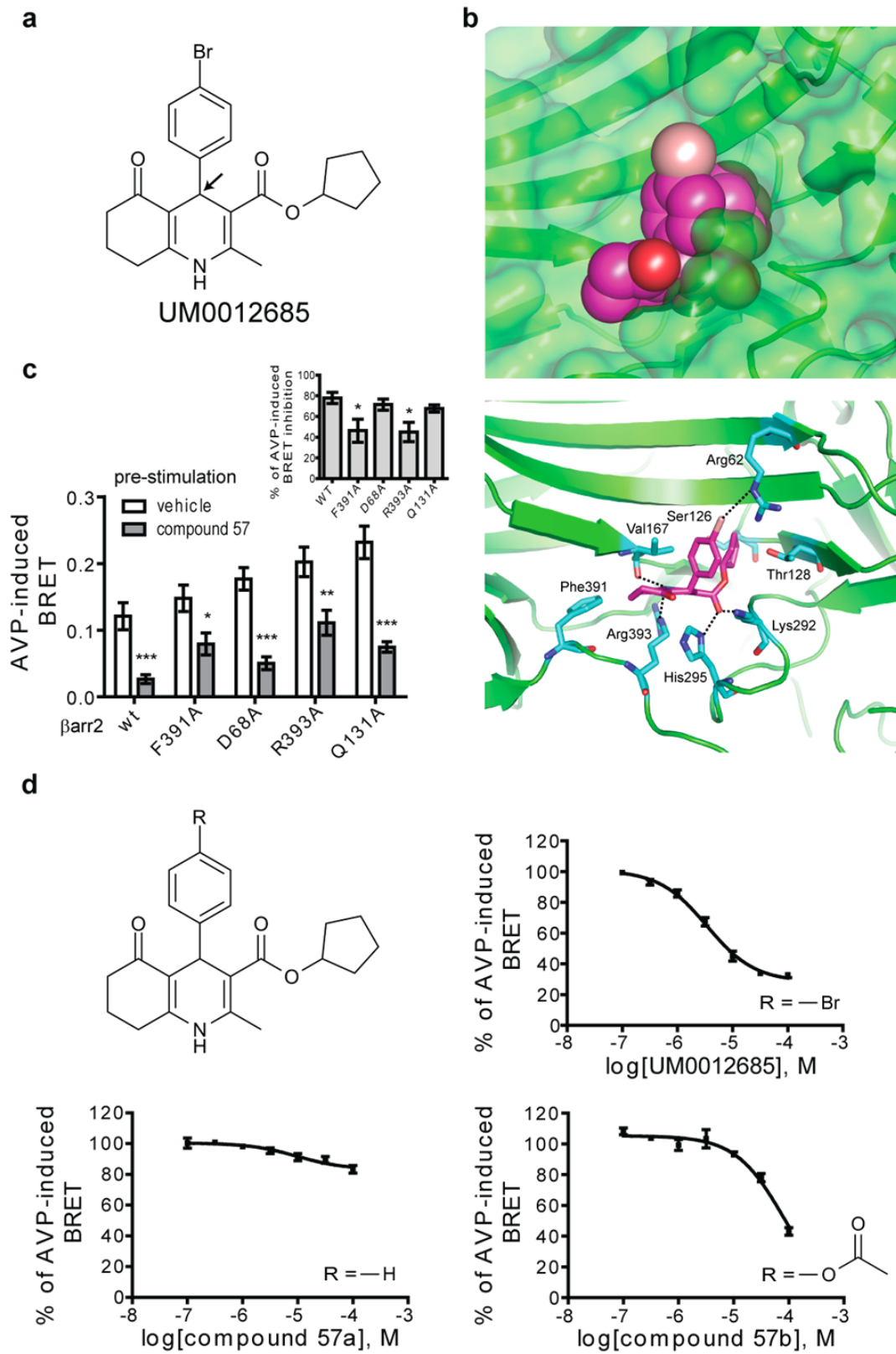


Figure 3. Validation of predicted UM0012685 binding mode. (a) UM0012685 two-dimensional structure. Arrow indicates chiral center on carbon 4 of the quinoline group. UM0012685 is cyclopentyl 4-(4-bromophenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinecarboxylate. (b) Predicted binding mode of UM0012685 on the polar core region of β arr1 model. Free binding energy predicted for S and R enantiomers are -6.53 and -5.39 Kcal/mol, respectively. S enantiomer (sphere or stick) docked on β arr1 model (ribbons overlaid with transparent surface or ribbons alone). β arr1 residues in close proximity of UM0012685 are represented as sticks. Dotted lines represent electrostatic interactions. Bromine, nitrogen and oxygen are colored in salmon, dark blue and red, while UM0012685 and β arr1 carbon atoms are colored in magenta and light blue, respectively. (c) AVP-mediated recruitment of β arr2 mutants to V2R detected by BRET. Cells transiently expressing V2R-eYFP and the indicated β arr2-RLucII mutants were pre-treated with UM0012685 (100 μ M, 25 min), then treated with AVP (100 nM, 5 min). Inset shows the percentage of BRET-signal inhibition. (d) BRET measured on the same cells as Figure 2d, pre-treated (10 min) with increased concentration of the indicated analogous compounds followed by AVP treatment (100 nM, 20 min). "R" indicates the position of chemical substitutions. $\log IC_{50} \pm$ s.e.m. are -5.5 ± 0.1 (UM0012685), -5.0 ± 0.3 (compound 57a), -4.1 ± 0.1 (compound 57b). Data are the mean $\pm$ s.e.m of 3 independent experiments. Significance against vehicle or wt- β arr2 (inset) was determined using two-tailed unpaired student t-test and ANOVA followed by Bonferroni's test, respectively (***P < 0.001; **P < 0.01; *P < 0.05).

FIGURE 4

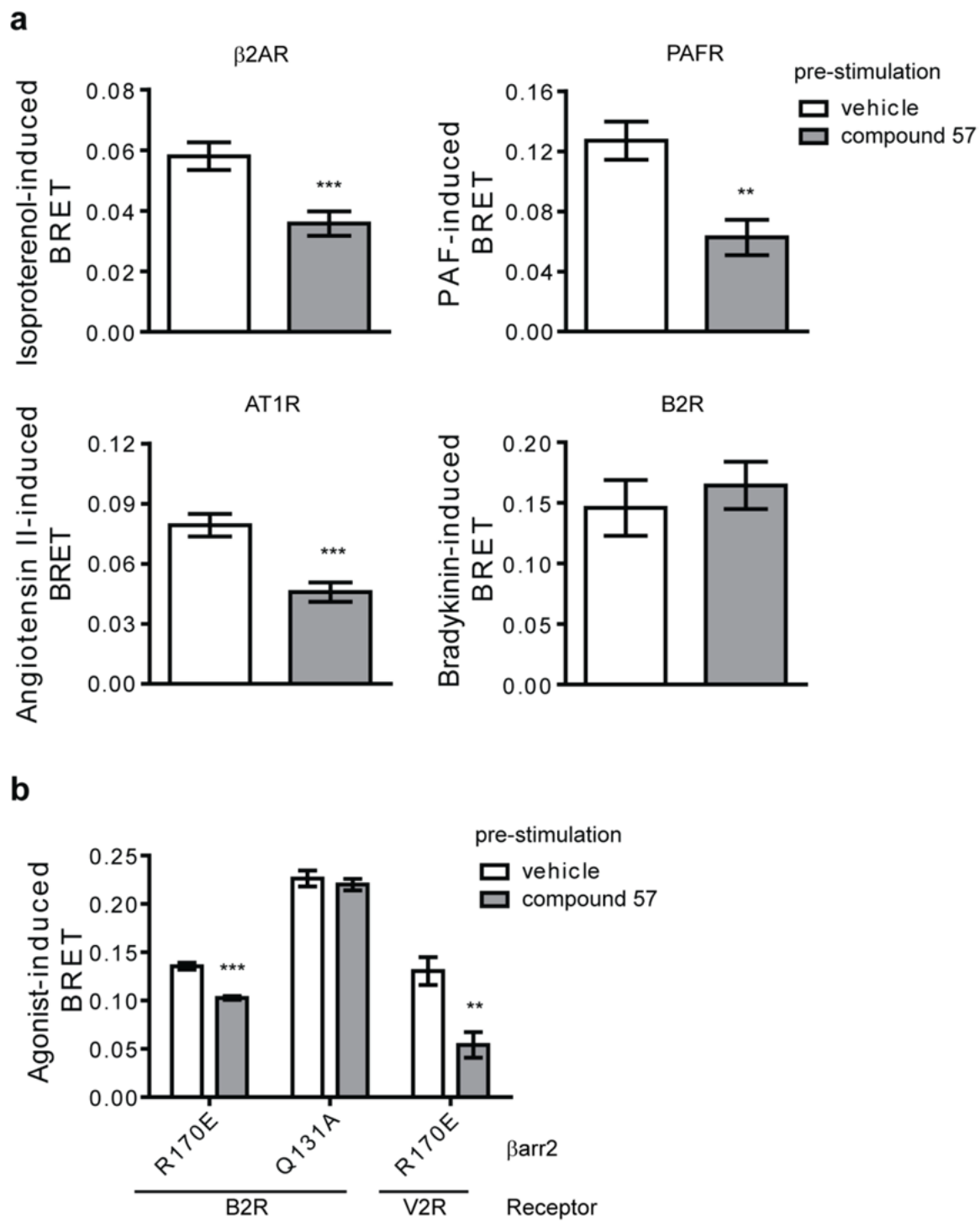


Figure 4. UM0012685 activity on β arr2 recruitment to several GPCR. (a) Cells transiently expressing one of the following receptors (β 2AR-YFP, PAFR-venus, AT1R-venus and B2R-eYFP) along with either β arr2-RLucI (with PAFR and AT1R) or β arr2-RLucII (with β 2AR and B2R) were pre-treated with UM0012685 (100 μ M, 25 min) followed by 5 min treatment with either isoproterenol (10 μ M), platelet activating factor (PAF) (100 nM), angiotensin II (1 μ M) or bradykinin (1 μ M), respectively. Agonist induced β arr2 recruitment was then measured by BRET. (b) Similarly to (a) but using B2R-eYFP or V2R-eYFP and the indicated variant of β arr2-RLucII. Data represent the mean $\pm$ s.e.m. of 3 to 7 independent experiments. Significance against vehicle pre-treated was determined using two-tailed unpaired student t test. (***P < 0.001; **P < 0.01).

FIGURE 5

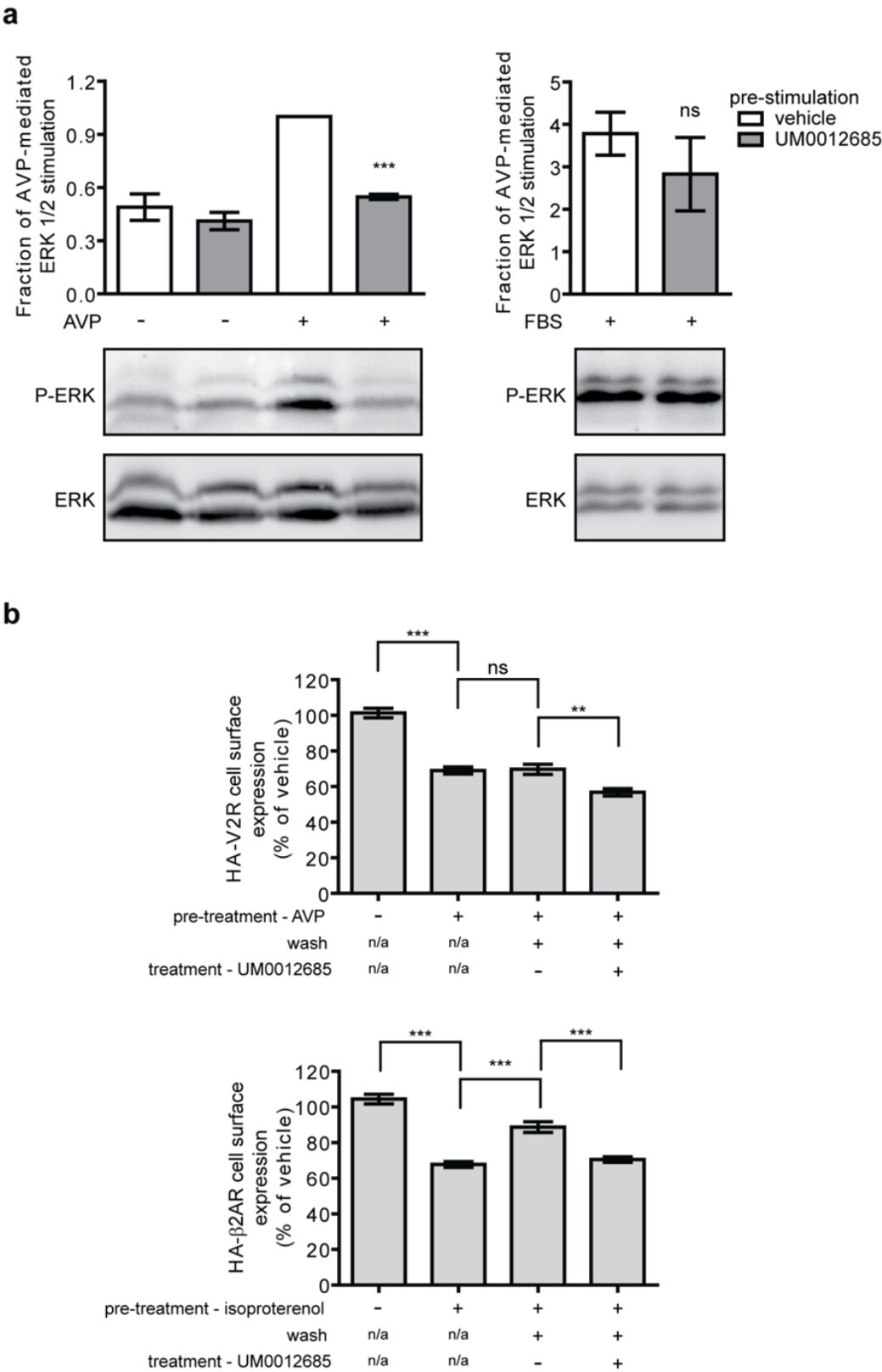


Figure 5. UM0012685 action on GPCR trafficking and signaling. (a) AVP-mediated ERK1/2 activation monitored by western blot. Same cells as Figure 2a,b,c were pre-treated with vehicle or UM0012685 (100 μ M, 10 min) , and then treated with vehicle, AVP (1 μ M, 2 min) or fetal bovine serum (FBS, 10%). Data represent the mean $\pm$ s.e.m of signal quantification for at least 3 independent experiments. (b) Cell surface receptors reappearance after agonist-stimulated receptor endocytosis (recycling) monitored by ELISA. Cells transiently expressing 3HA-V2R (upper panel) or HA- β 2AR (lower panel) were treated (30 min) with vehicle, AVP (100 nM) or isoproterenol (10 μ M) as indicated to induce receptor endocytosis. The samples were then washed extensively to remove any ligand and treated with vehicle or UM0012685 (100 μ M, 60 min). Data represent the mean $\pm$ s.e.m of 4 independent experiments. Significance for ERK1/2 experiment against vehicle pre-treated sample was determined using one sample t-test (AVP) or two-tailed unpaired student t-test (FBS). Significance for recycling experiment was determined using an ANOVA followed by a Tukey's tests. (***P < 0.001; **P < 0.01; ns = non-significant).

FIGURE 6

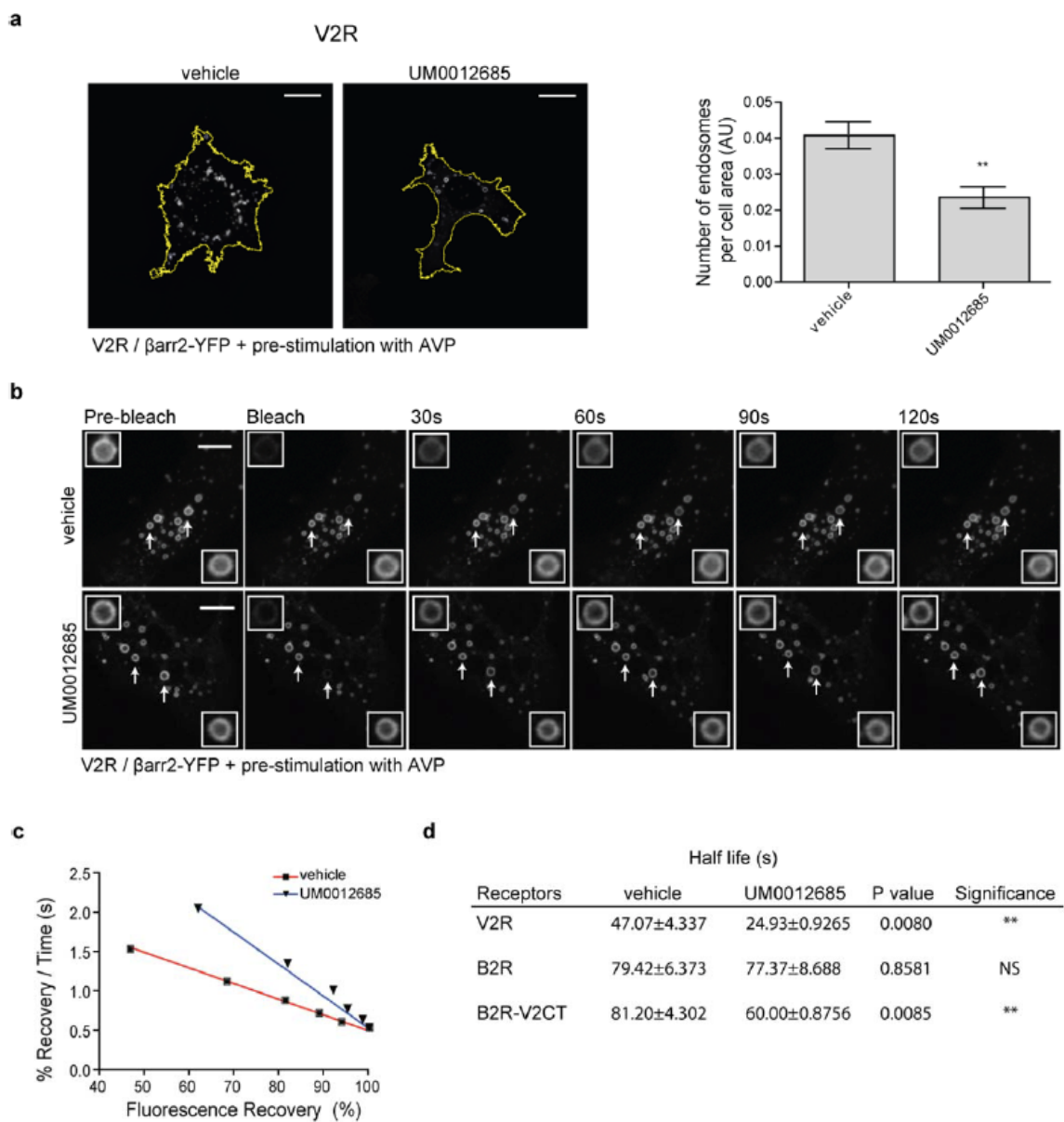


Figure 6. UM0012685 impact on β arr2/V2R complex lifetime in endosomes. (a) β arr2 localization in endosomes monitored by confocal microscopy. Cells transiently expressing HA-V2R and β arr2-YFP were pre-treated with AVP (1 μ M, 15 min), then treated or not (vehicle) with UM0012685 (100 μ M, 30 min). Micrographs are representative confocal images of YFP fluorescence where cell circumference is drawn in yellow. Bar graph shows endosomes density. Data are the mean $\pm$ s.e.m. of 3 independent experiments (15-22 cells). (b,c,d) Dynamic of β arr2 association with agonist-induced endosomes monitored by FRAP. Cells transiently co-expressing V2R, B2R, or B2R-V2CT along with β arr2-YFP were pretreated (15 min) with AVP or bradykinin (1 μ M), then treated or not (vehicle) with UM0012685 (50 μ M, 30 min). (b) Representative images of FRAP experiment for AVP pretreated V2R expressing cells, comparing vehicle and UM0012685 treatment. Immediately after UM0012685 treatment, endosomes were selected (arrows), and one was bleached. Fluorescence recovery rate of the bleached endosome was monitored every 30 sec for 2 min and compared to a non-bleached endosome. Top left inset box represents enlargement of the pre-bleached and bleached endosome. Bottom right box is the control unbleached endosome. Scale bar is 10 μ m. (c) Linear regression analysis of recovery rates from b. (d) Calculated half-life of β arr2 recovery on endosomes from agonist pre-treated V2R, B2R, or B2R-V2CT expressing cells. Data are the mean $\pm$ s.e.m. of at least 3 independent experiments, for 17-20 endosomes. Significance was determined using two-tailed unpaired student t-test. (**P<0.01; ns = non-significant).

Supplementary Data

Supplemental Experimental procedures

Membrane Preparation and Radioligand Binding Assay - HEK293 cells were mechanically lysed in hypotonic buffer (25 mM Tris, 2 mM EDTA and protease inhibitor pH 7.4) and centrifuged at 800 x g for 10 min at 4⁰C to get rid of the nuclei and cell debris. Supernatants were then centrifuged at 40,000 x g for 20 min at 4⁰C and the pelleted membranes washed twice in the same buffer prior to determining protein concentration using DCtm protein assay. Thirty or 100 µg of membrane proteins were then incubated in binding buffer (75 mM Tris, MgCl₂ 12.5 mM, 2 mM EDTA and protease inhibitor pH 7.4) with 100 nM of V2R agonist [³H]AVP in the presence of vehicle or indicated compounds for 90 min at room temperature. The binding reaction was stopped by rapid filtration of the membranes on GF/C fiber glass filters, separating free from bound [³H]AVP. Residual radioactivity on the filters was determined using scintillation counting.

Supplemental Figures and Legends

FIGURE S1

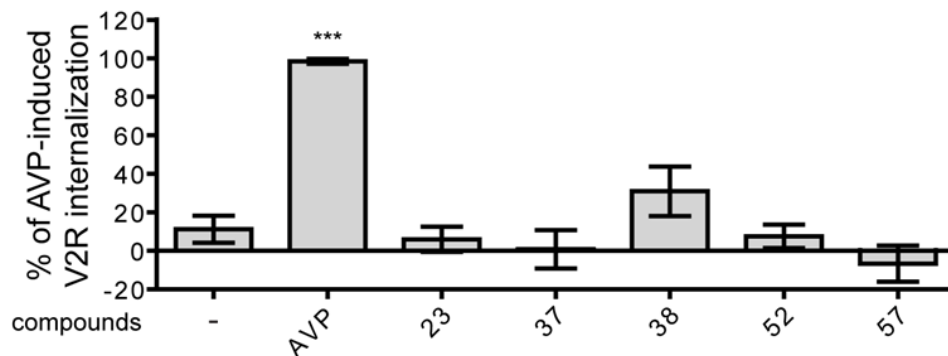


Figure S1. Modulation of V2R cell surface expression by candidates in the absence of AVP stimulation. FACS measurement of HA-V2R-venus cell surface expression on the same cells as Figure 2,a,b,c following stimulation with vehicle (-), the indicated compounds (100 μ M, 30 min) or AVP (100 nM, 20 min). The extent of endocytosis was determined relative to the AVP- and vehicle-stimulated samples as already described (see experimental procedures section). Data represent the mean $\pm$ s.e.m. of 3 independent experiments. Significance was determined using ANOVA followed by Bonferroni's test against vehicle-treated cells (*** $P < 0.001$).

FIGURE S2

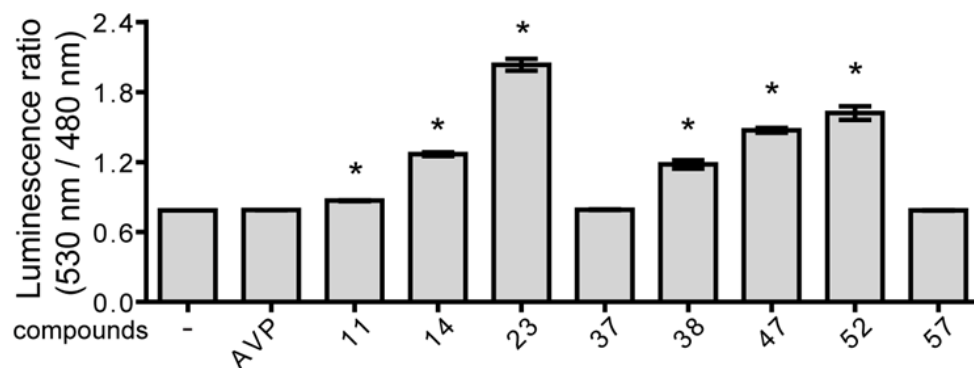


Figure S2. Candidate spectroscopic interference with luminescence signal. Luminescence ratio detected from cells stably expressing RLuc and treated (50 min) with vehicle (-), AVP (100 nM) or the indicated compounds (100 μ M). Data represent the mean $\pm$ S.E.M. of six independent experiments. '*' indicates deviation of the luminescence ratio outside the limit of ± 5 times the standard deviation of vehicle-treated cells.

FIGURE S3

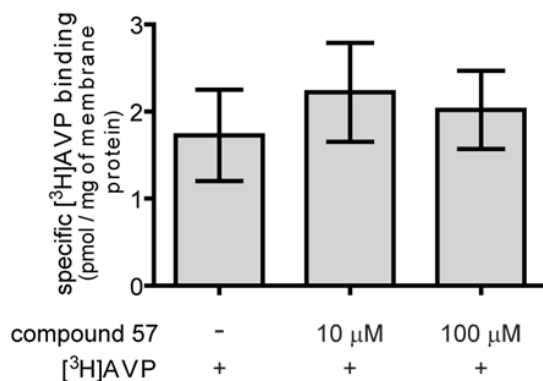
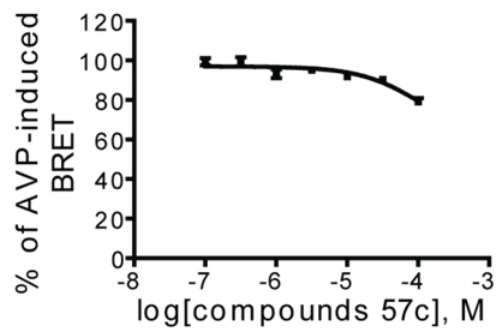


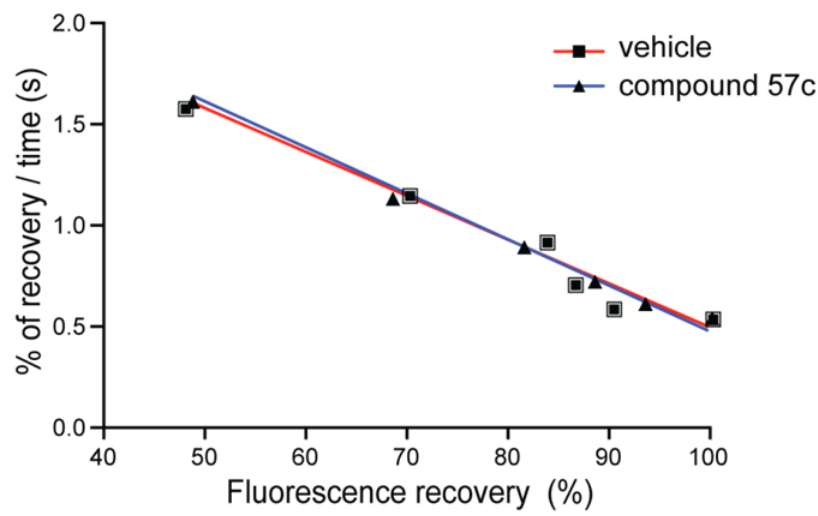
Figure S3. Compound 57 activity against AVP binding to V2R. [³H]AVP radioligand binding assay on membrane preparation from the same cells as Figure 2a,b,c. Membranes were pre-incubated (10 min) with vehicle or the indicated concentration of compound 57 followed by [³H]AVP incubation. Non-specific binding was determined by adding the V2R antagonist SR121463 (10 μM) to the samples at a concentration that displace 99.9% of AVP binding to the receptor and was subtracted from the total binding obtained for each sample to obtained the specific binding. HA-V2R-venus expression was determined to be 1.73 ± 0.67 pmol of receptor/mg of membrane protein and corresponds 17% of [³H]AVP total binding. Data represent the mean $\pm$ s.e.m of at least 3 independent experiments. Significant difference between total binding in all samples against non-specific binding was found using ANOVA followed by a Dunnett's test ($P < 0.05$).

FIGURE S4

a



b



c

Half life (s)	vehicle	compound 57c	P value	Significance
V2R	45.16±1.184	48.85±1.914	0.1997	ns

Figure S4. Compound 57c ability to modulate β arr2 recruitment to V2R and affect endosomal β arr2/V2R complex. (a) Assay as described in Figure 3d, but using compound 57c. (b) Assay as described in Figure 6b,c, but using compound 57c. (c) Calculated half-life measured in vehicle- and compound 57c-treated samples. Compound 57c is cyclopentyl 4-(3,4-dimethoxyphenyl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-3-quinolinecarboxylate. Data are the mean $\pm$ s.e.m. of at least 3 independent experiments for 17-20 endosomes. Significance was determined using two-tailed unpaired student t-test (ns = nonsignificant).

FIGURE S5

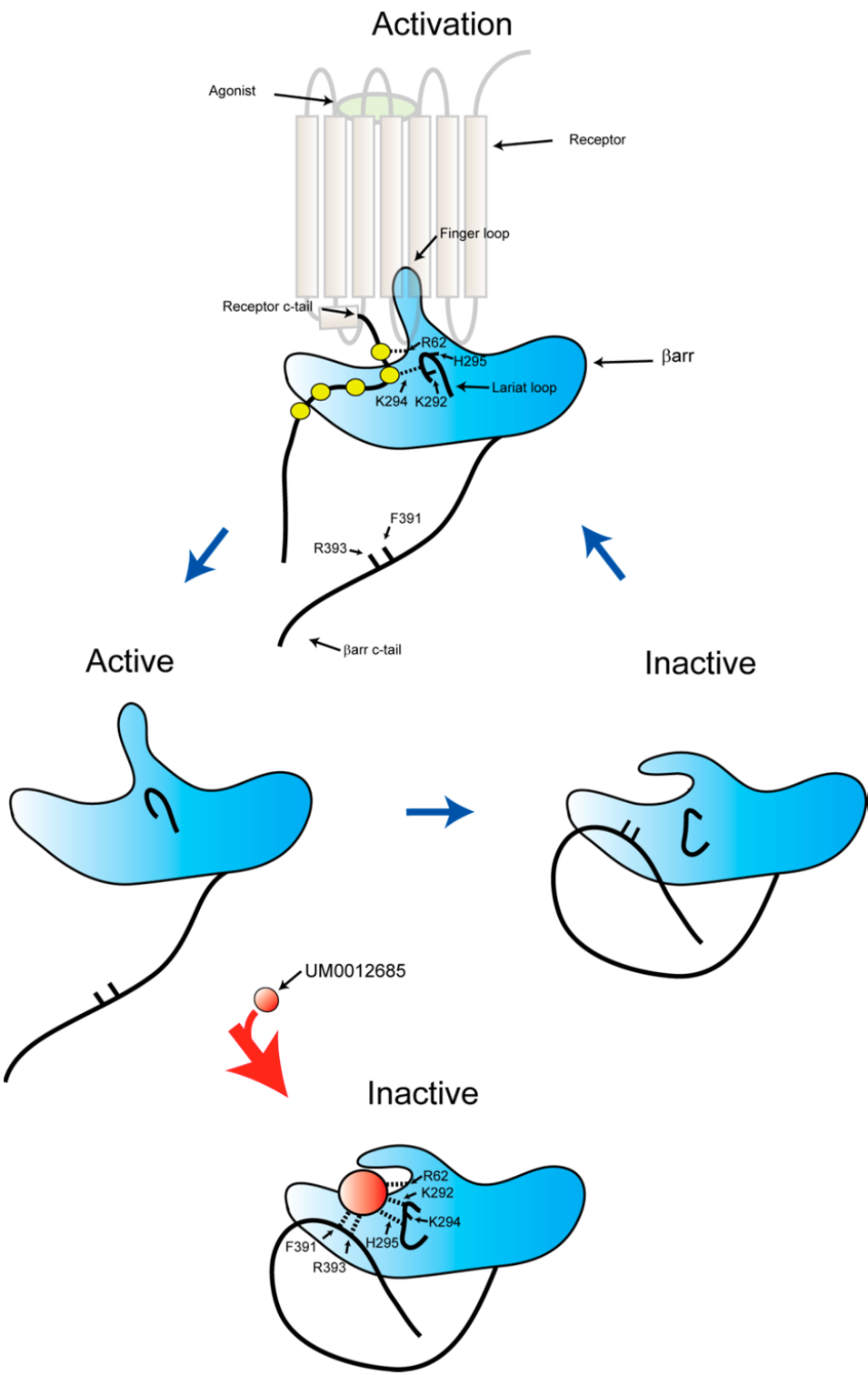


Figure S5. Model of UM0012685 mode of action on β arr. Hormone binding to a GPCR activates G protein signaling, resulting in G protein-coupled receptor kinases-mediated phosphorylation of the receptor inner domains. This trigger β arr recruitment and conformational changes that lead to its activation. UM0012685 is thought to stabilize the inactive basal state by competing directly with the receptor C-terminal tail, stabilizing a β arr C-tail position competing with receptor C-tail and a lariat loop conformation that disengage residues coordinating receptor C-tail phosphates. Black arrows indicate important structure element and residues. Dotted lines indicate residues interaction.

Chapter 5

Discussion and Conclusion

Discussion

The main goal of this work is to identify a new mode of regulation of β -arrestins in the endosomes and to study the role of arrestins structural domains in GPCRs trafficking and signaling. In the first study, our findings unveil a new signaling function of β -arrestin-2 by MAPK and highlights the role of the arrestin's hinge domain in the endosomal trafficking of GPCRs. The second study consists of a structure-based virtual screen of the arrestin and reveals for the first time a new pharmacological inhibitor of β -arrestin-2.

Acting as a multifunctional protein, the arrestin not only desensitizes and internalizes GPCRs, but also mediates several signaling responses in the cell, including MAPKs, which is the most studied cascade amongst all pathways. Serving as a scaffolding protein, others and we, have previously shown that β -arrestin regroups signaling effectors in the endosomes, such as Raf1, MEK1 and ERK2 (Tohgo, et al. 2002; Zimmerman, et al. 2011). However, the regulation of endosomal GPCR/ β -arrestin complexes and trafficking by MAPK was not yet addressed. Therefore, several assays, including pharmacological and non-pharmacological approaches, were set up to determine how such endosomal signaling function of β -arrestins would affect receptor trafficking. The inhibition of ERK1/2 activity, using either PD98059, PD184352 (pharmacological inhibitors of MEK) or MEK DN (dominant negative form of MEK-1), decreased the affinity binding state of β -arrestin-2 towards B2R in the endosomes, leading to a faster recycling of the receptor to the plasma membrane. Such consequence on receptor trafficking correlates with previous observations, showing that β -arrestin dissociation from B2R is necessary for receptor recycling (Simaan, et al. 2005).

On the other hand, blocking ERK1/2 activation did not impair the stability of B2R/ β -arrestin-1 endosomal complexes. Other than their cell localization, differences between β -arrestin-1 and β -arrestin-2 signaling functions remain ill studied. However, results obtained here and elsewhere (Lin, et al. 1999), strongly suggest distinct mode of regulation between both arrestins in endosomes. Notably, the affinity of β -arrestin-1 to B2R was lesser than β -arrestin-2, which led to the formation of weaker complexes with the receptor.

Sequence alignment analysis between β -arrestin-1 and β -arrestin-2 was performed, and interestingly, we found that only β -arrestin-2 had an exclusive ERK1/2 phosphorylation motif (PET¹⁷⁸P) in the hinge domain, in addition to a putative ERK1/2 docking site (K/R₂₋₃-X₁₋₆-L/I-X-L/I) (e.g. *161*-KR-NSVR-L-I-I-*169*). These findings suggest that β -arrestin-2 is a substrate of ERK1/2, potentially phosphorylated on Thr178 of the hinge domain, and such process is important in maintaining receptor/ β -arrestin endosomal complexes. In order to confirm that β -arrestin-2 is indeed phosphorylated by ERK1/2, we performed a complementary co-immunoprecipitation experiment, where phosphorylation levels of arrestin were detected using a specific antibody for phospho-Thr-Pro (Fig. 1). Beta-arrestin-2 phosphorylation was increased upon ligand-activation of B2R. Treating cells with PD98059 significantly decreased the arrestin phosphorylation state, and phospho-Thr-Pro levels were dramatically diminished when over-expressing the phospho-mimic form of β -arrestin-2 (β arr-2 T¹⁷⁸D). Because β -arrestin-2 sequence only contains two Thr-Pro residues (positions 178 and 276), the dramatic effect of T¹⁷⁸D mutant on arrestin phosphorylation suggests that Thr276 is not likely to be phosphorylated on the arrestin. A better inhibition of arrestin phosphorylation would have

been expected with PD98059 conditions, however, the effect can be underestimated due to several limitations of the experiment, such as the efficacy of the PD98059 to block ERK1/2 phosphorylation (Fig. 1a), which usually results in 50-60% inhibition at a concentration of 20 μ M. In addition, the specificity of phospho-Thr-Pro antibody can bring slight issues in terms of detecting the exact level of arrestin phosphorylation.

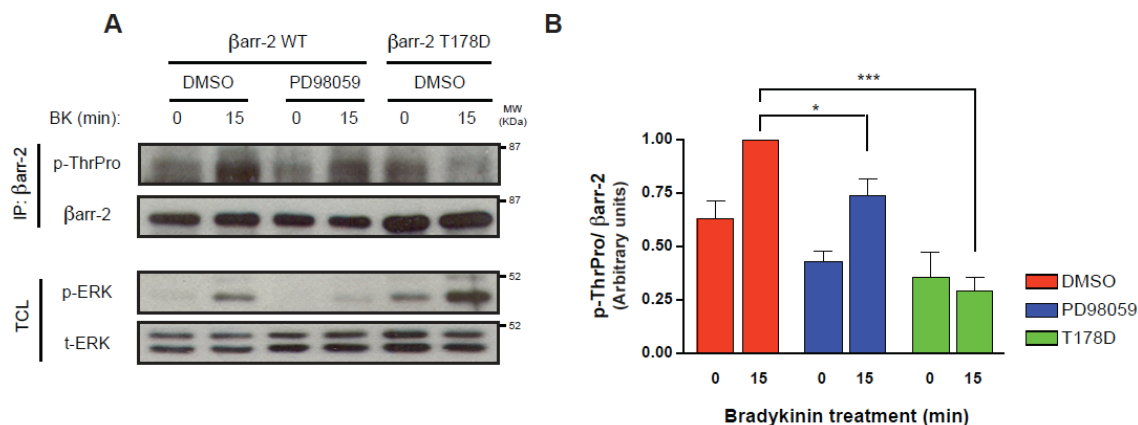


Figure 1. Phosphorylation of β -arrestin-2 on threonine 178.

HEK293 cells stably expressing HA-B2R were transfected with β -arrestin-2, either treated with DMSO or PD98059 (20 μ M, 30 min), or transfected with β -arrestin-2 T¹⁷⁸D. Cells were then challenged with bradykinin (1 μ M, 15 min). Lysates from immunoprecipitated (IP) Myc- β arr-2 were analyzed by western blot using anti-phospho-Thr-Pro and Myc antibody to detect phosphorylation levels and amounts of β -arrestin-2, respectively. Total cell lysates (TCL) were blotted for p-ERK and t-ERK. Shown are representative blots of three independent experiments. Molecular markers of 52 and 87 kDa are presented. (B) Densitometry analysis of immunoprecipitation experiments. Data represent the relative expression of phospho-Thr-Pro normalized to the amounts of Myc- β arr-2, as compared to the 15 min bradykinin stimulation, which is set at a 100% of the DMSO condition. Densitometry results are presented as the mean $\pm$ SEM. Statistical significance was analyzed by a two-way ANOVA followed by a Bonferroni post-test. *, $p < 0.05$; ***, $p < 0.001$.

Based on our findings, we proposed a model where MAPK targets β -arrestin-2 in the endosomes and maintains a stable complex between receptor/arrestin/ERK1/2, but it is still unclear how mechanistically the arrestin conformational rearrangements, induced by MAPK, is increasing the complex affinity. Many structural studies have revealed crystal structures of arrestins, demonstrating that the binding of the arrestin to the receptor requires a two step process of intramolecular interactions, namely the polar core and the ‘three-element’ interactions (Gurevich, et al. 2014; Shukla, et al. 2013). Upon ligand-activation of GPCRs, the phosphorylated tail of the receptor engages into the arrestin, destabilizes the polar core and the ‘three-element’ interactions, leading to the release of the C-tail of the arrestin and the closing of the N- and the C-terminal lobes in a ‘*clamshell*’-like conformation (Gurevich and Gurevich 2004). Based on this latter scenario, the hinge domain that connect the N- and C-terminal domains together have been shown to be crucial for stabilizing the receptor/arrestin complex interaction. However, no conformational changes of the hinge domain have been yet reported upon activation of the arrestin. Therefore, more work is needed in order to better understand the role of β -arrestin conformational structures in regulating the endosomal binding of the arrestin to the receptor. For instance, using BRET-based assays to detect conformational changes of β -arrestin, we recently revealed that angiotensin II biased analogs selectively engage distinct β -arrestin conformations, leading to specific signaling outcomes (Zimmerman, et al. 2012). Using a similar approach can thus be useful to detect conformational changes between β -arrestin-2 Thr178 mutants (T¹⁷⁸A or T¹⁷⁸D). A high resolution β -arrestin-2 crystal structure may also provide clues as to how the phosphorylation of the hinge domain would affect its conformation and hence increase

the binding state of receptor/arrestin complex. Here, we propose that the phosphorylation of the arrestin at Thr178 will provoke conformational rearrangements of the hinge domain, which in turn will destabilize the interactions of the Arg170 in the polar core and the ‘three-element’ network of electrostatic interactions. This latter process will thus promote a high affinity binding state of the arrestin to the receptor. By replacing the threonine by an aspartic acid residue, one would expect that such phospho-mimic form will mostly block the arrestin in a ‘super-active’ conformation. Indeed, β -arrestin-2 T¹⁷⁸D showed a two-fold increase in its affinity towards B2R, and this strong interaction was enough to trap the receptor in endosomes and prevent recycling. Moreover, several types of GPCRs were also investigated under these conditions, and introducing β -arrestin-2 T¹⁷⁸D mutant was enough to convert a class A receptor to a class B one, such as in the case of β 2AR, and increase endosomal receptor/arrestin complex stability in class B GPCRs, such as with AT1R and V2R. This general effect on GPCRs makes the “super-arrestin” mutant a valuable tool to better understand the trafficking behaviour of different types of receptors and further control the effect of G protein signaling on cellular responses.

One of the most studied arrestin-dependent pathways is the MAPK cascade. Characterized by a sustained signal in the cell, β -arrestin-mediated p-ERK1/2 activity has been shown to promote distinct biological responses, when compared to G protein signaling events (Tohgo et al. 2002). Here, we demonstrated that the aspartic acid mutant of β -arrestin-2 promoted a continued MAPK signaling, which was most probably caused by the long-lived endosomes containing receptor/ β -arrestin complexes. Because MAPK signaling is usually associated with several cellular responses, such as proliferation,

differentiation and cell survival, we sought to determine if the effect of β -arrestin-2 T¹⁷⁸D on MAPK would reach such important events in the cell. Indeed, the aspartic acid mutant of arrestin significantly increased proliferation upon ligand-mediated activation of B2R (Fig. 2). Unlike other studies showing that β -arrestins prohibit ERK1/2 from nuclear translocation, thus preventing *elk-1*-induced proliferation, our data suggest that the proliferative effect of the arrestin is due to the activation of other cytoplasmic targets, such as RSK1, 2, 3, known to induce proliferation through *c-fos* phosphorylation (Yoon and Seger 2006). However, the underlying mechanism of the β -arrestin-mediated MAPK cascade and its effect on proliferation remains elusive, and would require further investigations.

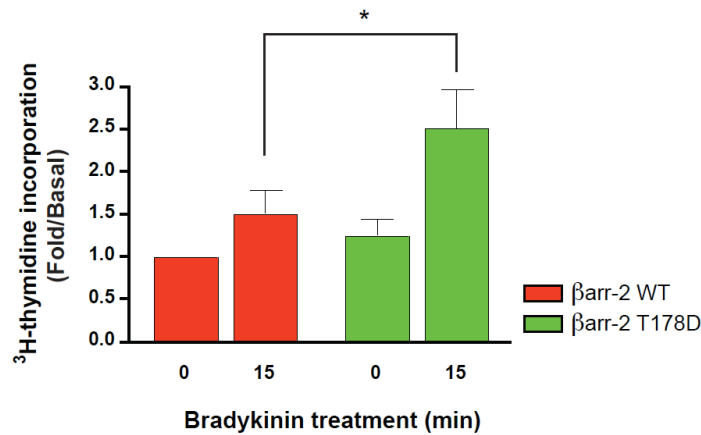


Figure 2. Effect of β -arrestin-2 T¹⁷⁸D on proliferation.

Cell proliferation was assessed by ³H-thymidine incorporation in COS-7 cells. Cells were transfected with HA-B2R, either β -arrestin-2 WT or β -arrestin-2 T¹⁷⁸D and serum starved for 48 hours. Cells were treated with BK for 8 hours then ³H-thymidine (0.5 μ Ci) was added to the milieu for 16 hours. Data represent thymidine incorporation ratio compared to untreated condition of β -arrestin-2 WT transfected cells, which is set to 100%. Results

are presented as the mean $\pm$ SEM of 8 independent experiments. Statistical significance was analyzed by a two-way ANOVA followed by a Bonferroni post-test. *, $p < 0.05$.

Other than showing differences between β -arrestin-1 and β -arrestin-2 in terms of receptor/ β -arrestin endosomal interactions, our results also revealed distinct behaviours between arrestins species. Human β -arrestin-2 showed a weaker affinity towards endosomal B2R than rat β -arrestin-2, and was insensitive to MAPK inhibition. This latter effect is most probably due to Lys178, a positive charged residue that is less probable to be targeted by ERK1/2. These findings suggest that in addition to MAPK, β -arrestins can be regulated by several other signaling effectors, which in one way or another can be essential for stabilizing the interaction between the arrestin and the receptor. For instance, the phosphorylation of β -arrestin-2 on Thr383 by casein kinase II and the ubiquitination of β -arrestin-2 on K11 and K12 showed to be involved in stabilizing receptor/arrestin complexes (Kim, et al. 2002; Shenoy and Lefkowitz 2005). Nonetheless, we do not think that ubiquitination is the alternative mechanism regulating human β -arrestin-2 on Lys178, because β -arrestin-2 mutant K¹⁷⁸A did not show any significant changes in the affinity between the arrestin and the receptor in the endosomes (data not shown). However, a gain-of-function in the endosomal B2R/ β -arrestin-2 interaction was observed when human β -arrestin-2 Lys178 was substituted with threonine (K¹⁷⁸T) and *vice-versa*. Moreover, in terms of trafficking and signaling outcomes, human β -arrestin-2 K¹⁷⁸D showed similar behaviours than rat β -arrestin-2 T¹⁷⁸D, suggesting that T/K178 of the hinge domain is a key residue in regulating the binding of receptor/arrestin complexes, whether by phosphorylating the threonine, such as in the case of rat β -arrestin-2 or using

another mode of regulation for the human β -arrestin-2. Therefore, an important aspect to consider when performing research studies in cells or in other type of systems is the protein species as well as its endogenous level of expression, which might highly influence the results. In our study, for instance, the MAPK putative site identified in the hinge domain was not conserved over species. Rat and mouse shared a Thr at the same position, whereas in bovine and human β -arrestin-2 it is replaced with a Lys instead. Phylogenetic studies always refer to changes in residue positions over species evolution, suggesting that an amino acid can move to a different region without modifying the protein function. Such modifications often occur to either phosphosites or ubiquitination sites specifically in disordered and flexible regions (Hagai, et al. 2012). However, despite the fact that arrestins hinge domain is a highly flexible and a well exposed region, we believe that it is not the case for β -arrestin-2, because no additional threonine was found in the human β -arrestin-2 when compared to the rat (Gnad, et al. 2007; Gurevich and Benovic 1997).

In summary, our findings highlight a new mode of regulation of endosomal GPCR/ β -arrestin-2 complexes and trafficking by MAPK. We also identified a novel regulatory site in the hinge domain of rat β -arrestin-2 and showed that phosphorylation of this target residue increased the stability of receptor/arrestin endosomal complexes, diminished receptor recycling and promoted distinct cellular signaling and biological responses.

Protein-protein interactions is a process involved in almost all signaling cascades in the cell. Therefore, having the ability to disrupt or enhance such interactions can selectively direct a signaling pathway over another (*e.g.* biased signaling); a concept that

was shown to be beneficial for the development of better therapeutic drugs. In order to induce a high affinity binding state of receptor/arrestin complexes, an interesting approach to use for our study would be through generating a monomeric single domain antibody (nanobody) that specifically targets residue 178 of β -arrestin-2. An example of such approach is the CXCR7-targeting nanobody therapy. A recent study reported beneficial effects of lama-derived single variable domains directed against CXCR7, including a reduced head and neck cancer cell growth *in vivo* (Maussang, et al. 2013). Thus, one would expect that introducing such nanobody against Thr/Lys 178 of β -arrestin-2 in cells would stabilize the interaction between GPCR and β -arrestin-2 in endosomes, and control receptor signaling, whether by preventing its recycling to the plasma membrane or engaging distinct intracellular signaling pathways leading to various physiological responses.

Another approach to regulate the binding of the arrestin to GPCRs is presented in Chapter 3. In this study, we used a virtual screen based on the crystal structure of β -arrestin-1 and identified for the first time a β -arrestin-2 inhibitor capable of diminishing receptor endocytosis, thus leading to distinct cell signaling outcomes. Until today, signaling functions of β -arrestins have been studied via several approaches, such as using either arrestin knockout mice, generating GPCRs mutations or introducing bias ligands that favor one signaling pathway over another. However, here we provide a new pharmacological tool as an alternative to study arrestins trafficking and signaling functions.

Based on the crystal structure of the inactive state of visual arrestin and β -arrestin-1, studies have reported many conformational rearrangements in different domains of the arrestin. Interestingly, it was noted that the highly flexible finger loop, located between β -strands V and VI was folded over the center of the arrestin, where it buries the phospho-sensor R175 in the polar core, thus blocking all access to this residue (Gurevich and Benovic 1997; Hanson, et al. 2006). Upon receptor activation, β -arrestin undergo conformational changes due to the binding of the phosphorylated C-tail to the arrestin which not only destabilize the intramolecular interactions from the polar core and the ‘three-element’ network but also induce interdomain rearrangements mostly detected in the arrestin loops (Shukla et al. 2013). Based on such conformational analysis, we thus used the inactive form of β -arrestin-1, which was the only crystal structure available during this study, as a prototype for the virtual screen; and provided a simple modification to the structure by changing the finger loop position to open the access to the polar core network. Over 30,000 compounds were evaluated on energy binding, affinity and cell-based assays, and amongst all molecules, one compound, namely UM0012685, was able to block at a high potency V2R endocytosis, reduce β -arrestin-2/receptor endosomal interactions, alter receptor recycling and promote distinct signaling responses in the cell. The virtual docking has predicted several sites in β -arrestin that were considered as potential targets of compound UM0012685. As expected, selected residues were either involved in the binding of the phosphorylated C-tail of the receptor to the arrestin, such as R62, or located in the C-tail and lariat loop of the arrestin, which are known to undergo major spatial movements upon receptor C-tail binding, such as F391, R393, H295, K292. Hence, we proposed that UM0012685 stabilizes the inactive

form of β -arrestin by competing with the phosphorylated C-tail of the receptor and preventing the release of the arrestin C-tail, which also blocks the access of the receptor C-tail to the polar core. Indeed, the structural binding of the compound to arrestin was demonstrated by several mutations assays. First, we showed that the inhibitory effect of the compound on β -arrestin-2 recruitment to V2R was reduced when cells were transfected with β -arrestin-2 mutants, either F³⁹¹A or R³⁹³A. Second, we showed that replacing the bromine moiety of compound UM0012685, which is expected to bind residue R62 of the arrestin, by a hydrogen, diminished the effect of the compound on arrestin recruitment, whereas replacing this residue with a methyl ester (similar binding properties as bromide) significantly blocked arrestin recruitment to V2R. The effect of UM0012685 on endocytosis was also tested for several other GPCRs, such as AT1R, PAFR, β 2AR and B2R. Compound UM0012685 blocked ligand-mediated endocytosis of all receptors except B2R. These results also correlated with the endosomal interaction between β -arrestin-2 and the receptor when evaluated by FRAP technique. However, using a chimera of the B2R bearing the V2R carboxyl terminal tail (B2R-V2CT) rescued the inhibitory effect of the compound, decreasing once again the affinity binding state of receptor/ β -arrestin-2 complexes in the endosomes. Contrarily to other tested receptors, because B2R can partially recruit arrestin independently of its C-tail phosphorylation (Feierler, et al. 2011), we think that this receptor might engage a distinct arrestin conformation that would alter the UM0012685 binding on the arrestin, and eliminate the effect on receptor endocytosis. Indeed, introducing a constitutively active form of β -arrestin-2 (R¹⁷⁰E), which is expected to bind UM0012685, showed a significant decrease in B2R endocytosis in the presence of the compound. Based on the docking prediction

analysis and in agreement with our results, compound UM0012685 is suggested to specifically bind the active form of β -arrestin-2, where the finger loop of the arrestin is maintained in an open conformation usually triggered by receptor C-tail.

We assessed the role of compound UM0012685 on V2R-mediated ERK1/2 activation. As expected, the UM0012685 significantly blocked ERK1/2 activity in AVP-stimulated V2R conditions with an IC_{50} of 13.7 μ M (Fig. 3C,D), whereas no effect was observed with B2R neither with prostaglandin F2 α receptor (FP) (Fig. 3A,B). Such a result was not surprising because we have already showed that PGF2 α -mediated stimulation of FP receptor does not induce β -arrestin recruitment, and demonstrated that PGF2 α -induced MAPK activation is a β -arrestin-independent signaling event (Goupil, et al. 2012). However, it has been previously shown that AVP-mediated ERK1/2 activation is dependent on the engagement of β -arrestin which promotes MAPK signaling pathway through IGFR transactivation (Charest, et al. 2007; Oligny-Longpre, et al. 2012). This latter scenario suggests that UM0012685 might inhibit MAPK activation through blocking the arrestin binding to IGFR and its signaling. The differential effect of this compound on V2R in contrast to other GPCRs should thus be addressed by testing the ERK1/2-mediated IGFR transactivation by these receptors. On the other hand, to prove if the V2R-mediated MAPK activation effect of the compound is a β -arrestin-dependent process, we tried to down-regulate β -arrestin-1/2 using shRNA targeting both β -arrestin isoforms. However, this approach could not be performed because knocking down the expression of β -arrestins in cells significantly decreased the ERK1/2 activity, thus making it impossible to evaluate the effect of UM0012685 on signaling. In addition to studying the receptor trafficking process, these findings also demonstrate that compound

UM0012685 is a useful tool to investigate arrestin signaling functions as well as their biological responses in the cell.

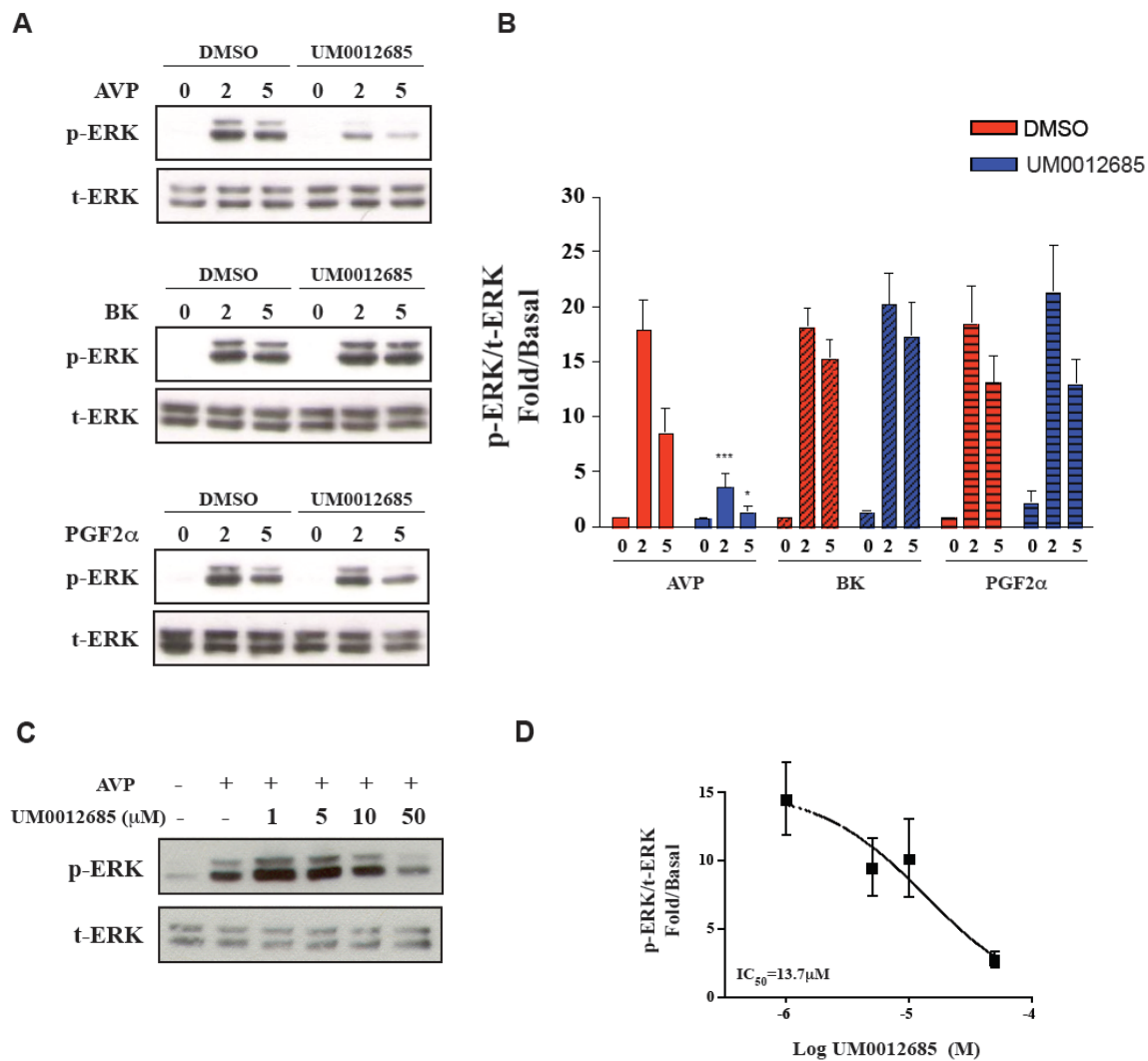


Figure 3. Effect of compound UM0012685 on GPCR-dependent MAPK activation.

(A, C) Shown are representative immuno-blots of ERK1/2 activation from HEK293 cells expressing either HA-V2R (A,C), HA-B2R or HA-FP. Cells were either treated with UM0012685 (50 μM; A) or in a concentration-response manner (1, 5, 10 and 50 μM; C)

for 10 min before being challenged with receptor corresponding ligands (1 μ M) for the indicated time. Lysates were immunoblotted for phosphorylated ERK (p-ERK) and total ERK (t-ERK). (B) Densitometry analysis of data from A, which represents the mean $\pm$ SEM of three independent experiments. p-ERK signals were normalized to that of t-ERK, and compared to non-treated (DMSO) cells for statistical analysis using a two-way ANOVA followed test followed by a Bonferroni post-test. *, $p < 0.05$; ***, $p < 0.001$. (D) Densitometry analysis from C plotted in concentration-response curve as fold over basal, and compared to non-treated (DMSO) conditions *versus* UM0012685 concentration. Results are representative of four independent experiments.

Beta-arrestin is known to control GPCRs trafficking, not only by inducing receptor internalization but also by playing an important role in receptor recycling (Simaan et al. 2005). Compound UM0012685 inhibited V2R and β 2AR endocytosis mainly due to an altered receptor/arrestin complex stability, but how is receptor recycling affected by such molecule was an intriguing question worth investigating. We thus evaluated receptor cell surface expression upon ligand removal, and showed that UM0012685 significantly inhibited β 2AR recycling. This suggested that the presence of β -arrestin with the receptor in the endosomes is crucial to support receptor recycling. Similar mechanism of action was also proposed for several other receptors, such as glucagon and CXCR7 receptors (Krillov, et al. 2008; Mahabaleshwar, et al. 2012). Until today, evaluating the effect of arrestins on receptors recycling remains a great challenge, because most studies tend to down-regulate the expression of β -arrestins in the cells. However, receptor endocytosis and recycling are generally altered when using such

approach. Here, we provide for the first time a useful tool to identify the role of β -arrestin-2 in GPCRs recycling. The V2R was also tested for recycling, however, this class B receptor did not show any recycling in the control conditions. Moreover, blocking receptor/arrestin interaction with UM0012685 did not restore V2R recycling to the plasma membrane. These observations confirm that the binding of β -arrestin to the receptor, whether is a weak or a strong interaction, is not exclusive to maintain the receptor in the endosomes, and that other mechanisms must be at play. Indeed, Arf (ADP-ribosylation factor) GAP (GTPase-activating protein) AGAP2, known to promote Rab4-dependent fast recycling of transferrin, was shown to interact with β -arrestin-1 and β -arrestin-2 and induce β 2AR recycling (Nie, et al. 2005; Wu, et al. 2013). Another example of this is Rab11a and Myosin Vb which are also known to regulate recycling of the M₄ Muscarinic receptor (M₄R) (Volpicelli, et al. 2002). In contrast, the dissociation of β -arrestin-2 from B2R was shown to be necessary for receptor recycling and resensitization (Simaan et al. 2005). This differential regulation of recycling between receptors support the fact that β -arrestin might not be solely responsible for controlling such process. For example, one would expect that blocking the interaction between β 2AR and β -arrestin-2 with the compound UM0012685 could disrupt the binding of AGAP2 to the endosomal complex which leads to the inhibition of receptor recycling. However, in the case of B2R, β -arrestin could compete with various recycling proteins in endosomes. Hence, the dissociation of the arrestin from the receptor could facilitate the binding of recycling adaptors, such as Rab11a, and promote the recycling of the receptor to the plasma membrane. The mechanism that regulates receptor recycling yet remains unclear,

therefore, more work is needed in order to highlight the differences in endocytosis and recycling processes between GPCRs.

Overall, this study demonstrated a novel approach of virtual screening, able to detect potential small molecules targeting β -arrestin-2 and regulating receptor trafficking and signaling. Here, the arrestin polar core was chosen as the screening platform, but it is well known that arrestin serves as a scaffolding protein that interacts with various adaptor proteins and signaling effectors. According to each binding partner, other virtual screens can also be designed in order to identify other type of molecules, which can reveal new signaling functions of arrestins. Indeed, a new study using a similar approach from Bouvier's laboratory has recently revealed a potential molecule targeting the interaction between AP-2 and β -arrestins.

Conclusion

During the last decade, GPCR ‘functional selectivity’ occupied great attention in the field of drug discovery. Opening many opportunities in terms of developing potent drugs, which represent higher level of selectivity and lower side-effects, ‘biased signaling’ was set as a new concept to treat diseases. GPCRs triggers a plethora of signaling cascades generally balancing between either G proteins, β -arrestins or other signaling pathways.

In this thesis, we mainly focused on characterizing the role of β -arrestin in regulating the trafficking and signaling of GPCRs. By identifying key residues in the hinge domain and the polar core of β -arrestin, our findings will help better understand the structural behaviour of the arrestin that is generally responsible for modulating its interaction with agonist-activated GPCRs. Enhancing or reducing the binding of β -arrestin to its receptor in the endosomes demonstrated remarkable effects on cell signaling responses, thus making it a valuable approach for applying biased signaling as a therapeutic benefit. Moreover, because β -arrestins endosomal signaling circuits induce a wide range of physiological responses that are different from those mediated at the plasma membrane, this scaffolding protein remains the perfect target for such approach.

Therefore, understanding the mechanism of trafficking and signaling of GPCRs will help, first, identifying signaling pathways induced by a specific receptor in a cell-/tissue- specific system and then associating each subset of signaling effectors to a disease state. Knowing the ‘signaling signature’ of a pathological condition or drug side-effects and having the ability to downregulate it would thus procure remarkable solutions for many treatments.

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