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Role of C-Terminal Tails of G Protein Coupled Receptors on Beta-Arrestin 1/2 Dependent Signaling

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by

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DEDICATION

To my grandparents

ABSTRACT OF DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, June 2013
Dr. Kathryn A DeFea, Chairperson

Protease activated receptor 2 (PAR2) and Neurokinin 1 receptor (NK1R) are 7 transmembrane receptors (7TMRs), which signal by $G_{\alpha q}$ leading to Ca^{2+} release and protein kinase C (PKC) activation. Both receptors are desensitized by β-arrestin binding to their C termini. They can also activate ERK1/2 through β-arrestin scaffolding complexes. They differ in ERK1/2 mediated physiological outcomes: cell migration versus proliferation. Using β-arrestins, PAR2 activates cofilin, to promote chemotaxis, which was not observed in NK1R. We hypothesized that the differences in β-arrestin dependent signaling by these 7TMRs can be attributed to how the molecular scaffolds bind the C-tail of the receptors. Using wild type and C-tail chimeras, we showed that the rate of desensitization, internalization, subcellular localization post endocytosis as well as ERK1/2 dependent physiological responses depend on the nature of interaction of β-arrestins with the C-termini. Bioluminescence resonance energy transfer (BRET) assays showed that PAR2 recruits both β-arrestin 1/2 faster and with

greater affinity than NK1R. We further show that initial $G_{\alpha q}$ signaling is necessary for β-arrestin 1/2 recruitment to NK1R. PAR2 recruits β-arrestin 1/2 even when $G_{\alpha q}$ pathway is blocked. Assays with C terminal phosphorylation mutants of PAR2 indicated that, phosphorylation of certain residues are necessary for β-arrestin 1/2 recruitment. Phosphorylation at putative PKC sites (S363 and T366) determine stability of PAR2-β-arrestin 1/2 complex. This in turn ensures downstream cofilin activation and cell migration. BRET assays with PAR2 and G-protein coupled receptor kinase-2 (GRK-2), revealed that GRK2 is recruited to PAR2 in a dose dependent fashion. It is possible that GRK-2 maybe another kinase which regulates PAR2 signaling. Finally, using allergic proteases from *Alternaria alternata* and *Blatella germanica* we demonstrated that PAR2 activation by these proteases leads to β-arrestin 1/2 recruitment and subsequent β-arrestin 1 dependent cofilin activation and cell migration. Cell migration brought about by the β-arrestin signaling arm of PAR2 might be an important molecular mechanism for migration of immune cells to the airways, which is an important symptom of allergic asthma.

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CHAPTER 1

Introduction

1.1 BACKGROUND

G protein coupled receptors (GPCRs) are the largest family of trans-membrane receptors, comprising nearly 4% of the human protein coding genome (1). They are found in most eukaryotes ranging from yeast to humans (2). GPCRs can mediate a multitude of functions ranging from vision, neurotransmission to cell migration. Very recently a number of nontraditional roles for GPCRs have been proposed like cytokinesis, autophagy and intracellular trafficking (3-5). Hence it comes as no surprise that nearly 40% of all prescription drugs target GPCRs(6).

The first GPCR to be crystallized was the bovine rhodopsin receptor followed by the human β_2-adrenergic receptor. Since then several other GPCRs like the β_1-adrenergic receptor, sphingosine-1-phosphoate receptor, D3 dopamine receptor, H1 histamine receptor, CXCR4 chemokine receptor and the adenosine receptor have been crystallized. The common structural theme that emerges from these discoveries is that GPCRs comprise of an extracellular N-terminus, which interweaves the membrane with seven transmembrane alpha helices and finally ending with a cytosolic C-terminal tail. These secondary structures are interconnected by, three extracellular and three intracellular loops (7-12). There have been reports about intramolecular disulphide linkages which enhances ligand binding and receptor activation (13). Several posttranslational modification associated, with GPCRs include phosphorylation, glycosylation, palmitoylation,

nitrosylation, ubiquitination which can regulate signaling by recruiting β-arrestins, promoting anterograde receptor trafficking from trans-golgi network, G protein coupling, receptor desensitization, endocytosis, receptor folding, ligand binding and lysosomal degradation (14,15) ((16-20).

- **1.1.1 Signaling by heterotrimeric G protein pathways:**

Following agonist binding, GPCRs are activated and signal by heterotrimeric guanine nucleotide binding proteins or simply, G proteins. G proteins comprise of α, β and γ subunits. The Gα subunit remains GDP bound in the inactive state (21). When GPCRs are activated by ligand binding, they undergo conformational changes in the transmembrane helices. This change in structure is more dramatic when transmitted to the intracellular loops. The arginine residue in the crucial D/ERY motif in the third transmembrane helix is linked to a tyrosine residue. This rearrangement establishes the binding surface with G proteins (22-24). This activates the G proteins and the Gα subunit exchanges the GDP for GTP [Figure 1.1]. This exchange process can be catalyzed by Guanine nucleotide exchange factors (GEF). Ligand binding to mammalian GPCR alters the conformation such that it can function as a GEF, to facilitate GDP exchange for inactive Gα (25). The active Gα-GTP dissociates from Gβγ subunits [Figure 1.2]. Both Gα-GTP and Gβγ subunits activate downstream effectors (26). A list of known effectors of the heterotrimeric G proteins has been provided in Figure 1.3.

Duration of Gα signaling is regulated by, a family of proteins called regulators of G protein signaling (RGS). Guanine nucleotide dissociation inhibitors (GDI) inhibit the initial exchange of GDP for GTP, which is necessary for activation of heterotrimeric G proteins (27). GTP bound to Gα is hydrolyzed by GTPase action of the G protein. This can be accelerated by "GTPase activating protein" or GAPs, which also belong to the RGS family (28). Following GTP hydrolysis, the Gα-GDP then gets re-associated with the Gβγ subunits to attain the inactivated state (27) [Figure 1.2].

Certain GPCRs like β2-adrenergic receptor, α2A-adrenergic receptor, human cannabinoid receptor (CB1), Protease Activated Receptor 1 and 2 (PAR1 and PAR2) are known to signal by multiple G protein subtypes. In case of the β2-adrenergic receptor, the switch from the prototypic Gαs pathway to the Gαi/o pathway is dependent on protein kinase (PKA) mediated phosphorylation of specific S/T residues in the C-tail of the receptor. This switch is completely abolished when the cells were pretreated with pharmacological inhibitor of PKA or in case PKA mutant β2-adrenergic receptor. In case of the α2A-adrenergic receptor the choice between the G proteins is dependent on the ligands used to stimulate the receptor. The CB1 receptor can also signal by Gαs or Gαi/o. The choice between the two pathways lie in the crucial Leu-222 residue in the conserved $DRY(X)_5PL$ motif of the second intracellular loop (IL2). Also in case of PAR1, structural determinants in the IL2 are essential for the choice of G

protein pathway. Five amino acids in the IL2 are essential for PAR-1 Gαq coupling. Mutation of one of these 5 residues, i.e., Arg-205 in the IL2 inhibits direct binding of PAR1 to Gαq. However this does not affect binding of the receptor to Gαi/o or Gα12/13 (29-34).

- **1.1.2 Termination of G protein signaling- Role of serine/threonine kinases:**

Signaling by the G protein pathway is terminated, when ubiquitously expressed cytosolic scaffolding molecules β-arrestin1/2 are recruited o the C-terminal tail of the receptor to mediate receptor desensitization and endocytosis (35). The classic paradigm for GPCR desensitization suggests that activated GPCRs are phosphorylated at multiple S/T residues in the C terminal tails or the second intracellular loop. This phosphorylation step is usually mediated by second messenger kinases like Protein kinase A (PKA) or Protein kinase C (PKC)(36,37).

Another group of S/T kinases called G protein coupled receptor kinases (GRK) comprises of a family of seven kinases named GRK1-7. GRK-1 (rhodopsin kinases) and GRK-7 (cone opsin kinase) are exclusively expressed in the photoreceptor cells and mediate phosphorylation of the rhodopsin receptor. GRK-4 also has tissue specific distribution, as it is largely expressed in high quantities in the testis. GRK-2 and 3 also known as β-adrenergic receptor kinase-1

and 2 (βARK-1 or 2) phosphorylate the β-adrenergic receptor. GRK2, GRK3, GRK5, GRK6 are more ubiquitously expressed in a variety of tissue types. Since only four GRKs have wide tissue distribution and there are several different types of GPCRs, it is expected that there are overlaps among these four enzymes for receptor substrates (38). While GRKs exclusively mediate homologous desensitization of agonist occupied receptors only, second messenger kinases carry out heterologous desensitization (39).

There are differences in the mechanisms by which the different GRKs are targeted for trafficking to the membrane. GRK-4 and GRK-5 are palmitoylated at their C-terminal end. Thus they are constitutively embedded at the membrane. GRK-1 and GRK-7, have a C-terminal CAAX motif, which is farnesylated following light stimulation. This lipid modification is essential for membrane targeting. GRK-2 and GRK-3 have C-terminal Gβγ binding domains. Thus, following the dissociation of GTP bound Gα from heterotrimeric G proteins, Gβγ subunits bind to these isoforms, for membrane recruitment. GRK-6 binds to negatively charged phospholipids in the membrane by polybasic peptide domains in it (40).

- **1.1.3 β-arrestin1/2 as signal terminators**

The two cytosolic multifunctional adaptor proteins β-arrestin1/2 play vital roles in terminating G protein dependent signaling by GPCRs. Phosphorylation of

C-tail of GPCRs is crucial for enhancing β-arrestin1/2 binding to GPCRs (41). Structurally both β-arrestin1/2 are composed of an N-terminal and a C-terminal domain, each made up of seven β-pleated sheets. The N terminal domain is essential for activated receptor recognition and the C-terminal domain for secondary receptor recognition. In between the two domains, lies the polar core containing the phosphate sensor, which remains in an inactive conformation by ionic interactions with the C-terminal domain. Using a series of point mutations, the Arg-175 residue in the visual arrestin molecule was identified as the phosphate sensor. In the basal state a salt bridge connects the Arg-175 with Asp-296. However, the crucial Arg-175 remains buried in deep in the polar core of the molecule and it appears that a number of other intramolecular interactions need to be disrupted to release this phosphate sensor.

Hence, β-arrestin1/2 binding to C–tail of phosphorylated GPCRs is thought to be a dynamic multistep process. It has been proposed that the Lys-14 and Lys-15 in the N-terminal domain of the molecule first recognizes the phosphorylated residues in the receptor and causes one of them to flip over. This interaction distorts the β-arrestin1 molecule. As a result, the lys-14 and 15 residues are pushed towards another β-pleated sheet containing a number of potential phosphate binding residues like (Lys166, Arg171, Arg175, and Lys176). This leads to breaking of the salt bridge between Arg-175 and Asp-296, thus releasing the phosphate sensor to bind to the receptor. Finally, the C-tail of the β-arrestin

molecule folds out (42-48) [Figure 1.5]. Binding of β-arrestin1/2 to the cytosolic face of GPCRs generates steric hindrance and competes with the G protein for the receptor. Thus, β-arrestin1/2 can be considered as signal terminators, as they shut down signaling by the classic G protein pathway.

Apart from uncoupling the GPCRs from the cognate G proteins, β-arrestin1/2 can also internalize these receptors by packaging them in endocytotic vesicles. β-arrestin1/2 can scaffold clathrin and adaptor protein-2 (AP-2) to promote trafficking of the desensitized receptor from the membrane into the cytosol (49,50) [Figure 1.4]. Certain post-translational modifications in β-arrestin1/2 can affect receptor internalization. β-arrestin-1 has potential phosphorylation site at S-412. Cytoplasmic β-arrestin-1 is phosphorylated and the potential candidate for this phosphorylation event is ERK1/2. This phosphorylated β-arrestin-1 is translocated to the membrane, which can mediate desensitization. However, β-arrestin-1 needs to be dephosphorylated to bring about internalization (51,52). On the contrary, β-arrestin-2 does not undergo phosphorylation. Instead the ability of β-arrestin-2 to internalize some receptors is dependent on its ubiquitination status. β-arrestin-2 is ubiquitinated following binding to β2-adrenergic receptor and this post-translational modification is essential for receptor internalization (53).

- **1.1.4 Trafficking pathways for internalized GPCRs**

Once internalized, the receptors can be either degraded in the lysozome or recycled back to membrane. This choice can be directed by some other accessory proteins, involved in cellular trafficking. Rab GTPases are crucial for receptor endocytosis trafficking. Rab5 internalizes β2-adrenergic receptor sorts them to early endosome. It prevents fusion of endosomes containing AT1R with lysosomes, to prevent degradation of the receptor. Rab4 has also been shown to be involved with receptor recycling and Rab11 brings about lysosomal sorting (54-56). Apart from GTPases, the ATPase N-ethylmaleimide sensitive fusion protein (NSF) has also been implicated in GPCR trafficking. Binding of NSF to the extreme C tail of β2-adrenergic receptor, not only ensures receptor internalization, but also brings about recycling back to the membrane (57). Using siRNA experiments in cultured cell lines, Sortin nexin-1 (SNX-1) has been shown to be involved with lysosomal trafficking of internalized PAR-1 from endosomes (58,59). Na^+/H^+ Exchange regulatory factor (NHERF) is another protein involved with receptor sorting post internalization. It binds to β2-adrenergic receptor C-tail by its PDZ domain and ensures receptor recycling. Any disruption of this interaction between the above mentioned proteins favor lysosomal trafficking over recycling. NHERF binding to C-tail of parathyroid hormone receptor, P2Y1 receptor and κ-opiod receptor has been reported for receptor trafficking as well (60-63).

- **1.1.5 β-arrestin1/2 as signal transducers**

Over the past decade or so there has been a paradigm shift in the field. β-arrestin1/2 are now considered signal transducers as well. Apart from GPCRs, β-arrestin1/2 also have number of non receptor binding partners, some of which are important signaling molecules belonging to the mitogen activated protein kinase pathway (MAPK), phosphatidylinositol kinase pathway (PI3K), nuclear factor κ-B (NF-κB) and others (64-66).

β-arrestin 1 and 2 utilize their highly flexible scaffolding surface to bind to these signaling molecules to bring about a multitude of physiological effects extending from the membrane to the nucleus. A phospho-proteome and bioinformatics analysis of the β-arrestin dependent signaling downstream of the angiotensin II type 1A receptor, identified several binding partners of these versatile scaffolding molecules which includes proteins involved in PI3K/Akt signaling, MAPK kinase signaling, regulation of cell cycle as well as cytoskeleton reorganization (67) [Figure 1.6].

β-arrestins 1 and 2 have been reported to activate ERK1/2 by scaffolding its upstream activators Raf and Mek. In case of many GPCRs β-arrestin1/2 are known to spatially restrict the activated ERK1/2 to specific cellular compartments like the membrane and cytoplasm. Downstream of AT1R, β-arrestin2 can promote chemotaxis by activating p38 MAPK (68). AT1R can also target filamin A,

through a receptor- β-arrestin-ERK1/2 complex. Filamin A binds to actin bundles to generate membrane ruffling in Hep-2 cell lines (69). Activation of the AT1R can also enhance translation rates by β arrestin dependent ERK1/2 activation, which in turn phosphorylates MAP kinase interacting kinase 1 and eukaryotic translation initiation factor 4E (70). β-arrestin 2 has been shown to scaffold Ask, MKK and JNK3 to bring about phosphorylation of JNK3. As in case in β-arrestin1/2 dependent ERK1/2 activation, JNK3 is also restricted to the cytosol. However, there are no known cytosolic targets of JNK3(71).

In addition to regulating signaling pathways in the cell cytoplasm, there is growing evidence that β-arrestin1/2 can also regulate transcription downstream of GPCR activation. β-arrestin1/2 are negative regulators of NF-κB dependent transcription. β-arrestin 2 can scaffold I-κB at its N-terminus and β-arrestin1/2 can bind to I-κB kinase and I-κB inducing kinase. Following β2-adrenergic receptor activation, the binding affinity of β-arrestin 2 for I-κB is enhanced. In addition to I-κB, β-arrestin 2 can simultaneously scaffold I-κB kinase. As a result I-κB cannot be phosphorylated by I-κB kinase for proteasomal degradation. In this way arrestins can negatively regulate NF-kB signaling, to modulate immune responses (72). β-arrestin 2 negatively regulates β-catenin signaling downstream of dopamine receptor D2. Following D2 receptor activation, β-arrestin 2 scaffolds PP2A, Akt and glycogen synthase kinase-3 (GSK-3). In this signalosome, PP2A dephosphorylates Akt to inactivate it. Thus, Akt mediated phosphorylation of

GSK-3 is abolished. Active GSK-3 goes on to phosphorylate β-catenin, which is eventually degraded (73).

β-arrestin1/2 also serves to transduce signals from GPCRs at the membrane into the nucleus. At resting conditions β-arrestin-2 is exclusively found in the cytoplasm, while β-arrestin1 is distributed both in the cytoplasm as well as the nucleus. The N-terminal domain is crucial for nuclear translocation of both the arrestins. β-arrestin-2 has the conserved leucine rich nuclear export signal (NES) at the C terminal end (74,75). It has been shown that Mdm-2 and JNK are distributed in the cytoplasm due to the nucleocytoplasmic shuttling of β-arrestin-2. In the nucleus β-arrestin-1 acts a co-activator as it forms a complex with transcription factors cAMP response element binding protein (CREB) and p300 to bring about transcription initiation at FOS promoters (76).

- **1.1.6 Biased signaling by GPCRs**

Over the years another paradigm shift in the field of GPCR research has been discovery that receptors can signal by the unique β-arrestin signaling arm, independently of the traditional G protein pathway. This phenomenon was first reported in AT1R. It was demonstrated that β-arrestin-2 was successfully recruited to a mutant AT1R (DRY/AAY), which was incapable of coupling to Gαq. The mutant receptor also activated ERK1/2 through a β-arrestin1/2 dependent pathway, which was 50% of the response observed in the wild type.

This β-arrestin-1/2 dependent response persisted on inhibiting PKC using pharmacological inhibitors. The ability of AT1R to signal independently of the G proteins was further supported by the fact that the wild type receptor was able to generate only 40% of ERK1/2 activation response when pretreated with the PKC inhibitor (77). Similar reports were also published downstream of parathyroid and β2-adrenergic receptor as well (78,79).

Such G-protein independent but β-arrestin-1/2 dependent pathways have been reported downstream of PAR-2. Using a variety of cell based and vitro assays, it has been shown that these pathways promote ERK-1/2 activation, cofilin dephosphorylation as well as PI3-kinase and AMP-kinase inhibition to bring physiological responses (66,80-82).

This existence of two completely independent signaling arms downstream of an activated GPCR, brings us to the concept of biased agonists. "Biased agonism" refers to the ability of a GPCR ligand to preferentially trigger or suppress a part of the receptor's downstream signaling pathways. Initially, this selective property of biased agonists had been restricted to a choice of different heterotrimeric G proteins. However published data have now extended the phenomenon of biased agonism to a choice between the classic G protein pathway or the β-arrestin pathway (83-89). This novel concept in GPCR pharmacology will help to design more effective drugs with fewer side effects [Figure 1.7].

In fact a number of drugs preferentially activating either the G protein pathway over the non-cannonial β-arrestin signaling arm or vice-versa are currently in cilinical trial. An analogue of the Angiotensin II peptide [Sar(1),Ile(4),Ile(8)]AngII (SII)] has been shown to phosphorylate the AT1R, recruit β-arrestin, promote receptor internalization and ERK1/2 activation without activating G protein during the course of its action (86). TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH) completely antagonizes the canonical G protein pathway and preferentially promotes β-arrestin signaling. In vivo testing of this biased agonist, has shown that it selectively triggers β-arrestin dependent effects like reduction in blood pressure and increase in cardiac output (90).

Apart from drugs, which promote biased agonism/antagonism in GPCRs by binding to the orthosteric ligand binding sites, pepducins can carry out biased signaling allosterically. Pepducins are short peptides, which are identical to the third intracellular loop of GPCRs with a lipid modification, which allows them to be membrane permeable. They compete with the G protein binding sites on GPCRs. As a result they can interdict signaling through G proteins, thus creating a signaling bias towards the β-arrestin pathway (91,92).

A better understanding of the regulation of these pathways is essential for manipulating GPCR responses in physiological systems. Using two prototypical GPCRs: PAR2 and NK1R we have compared and contrasted how the β-arrestin signaling arms differ. Through our experiments we have shown that the two

receptors differ in the duration of the initial G protein signaling, rate of internalization, post endocytotic sorting and β-arrestin dependent pathways. Generating C-terminal chimeras by switching the C–tails of the receptors to the cytosolic end of the seventh intracellular loops, we showed that all of the above mentioned properties of the receptors, follow a trend similar to that demonstrated by the C-terminal parent. This leads to the conclusion that β-arrestin dependent functions are directed by their interaction with the receptor C-terminal tails.

Furthermore, using different C-terminal phospho-mutants we showed that, there exist specific functional roles for each of these residues, in the regulation of β-arrestin-1/2 recruitment, binding and consequently downstream signaling pathways and the ultimate physiological outcome in cells. This fits in very well with the emerging concept of phosphorylation bar-code in the field.

Finally, pathologically relevant allergic proteases from the fungus *Alternaria alternata* and the household pest *Blatella germanica* were also shown to trigger β-arrestin-2 dependent cofilin signaling by activating PAR2. These proteases trigger early symptoms of allergic asthma (93). Hence, a better understanding of the role of PAR2's divergent signaling arm at the biochemical and molecular level in the progression of the disease is essential for developing agonists or antagonists for therapeutic purposes.

1.2 FIGURES & LEGENDS

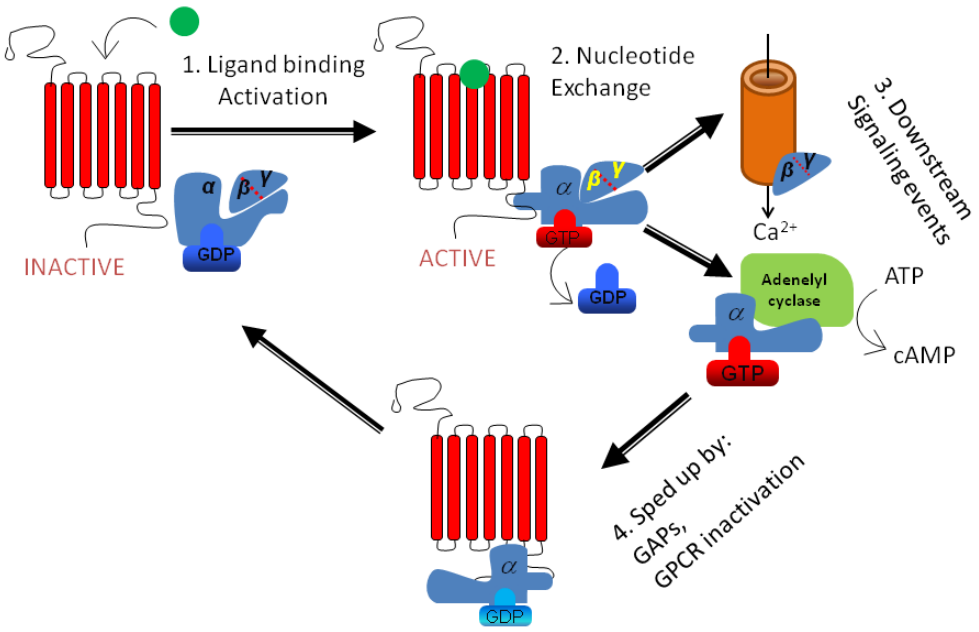

Figure 1.1: Activation of G protein coupled receptors:

Following binding of agonist to the extracellular domain of GPCRs, a change in the conformation of the cytoplasmic ends of the transmembrane helices takes place. This allows the activated receptor to bind to inactive heterotrimeric G protein complexes (Gα, Gβ and Gγ). Following association with the receptor, the GDP molecule bound to Gα subunit is exchanged for GTP. This step is followed by the dissociation of Gγβ subunits from the active receptor-Gα-GTP complex. Both the Gα and the Gγβ subunits can activate downstream effectors. Following GTP hydrolysis, the Gα-GDP subunit reassociates with the Gβγ complex to form the heterotrimeric complex (94).

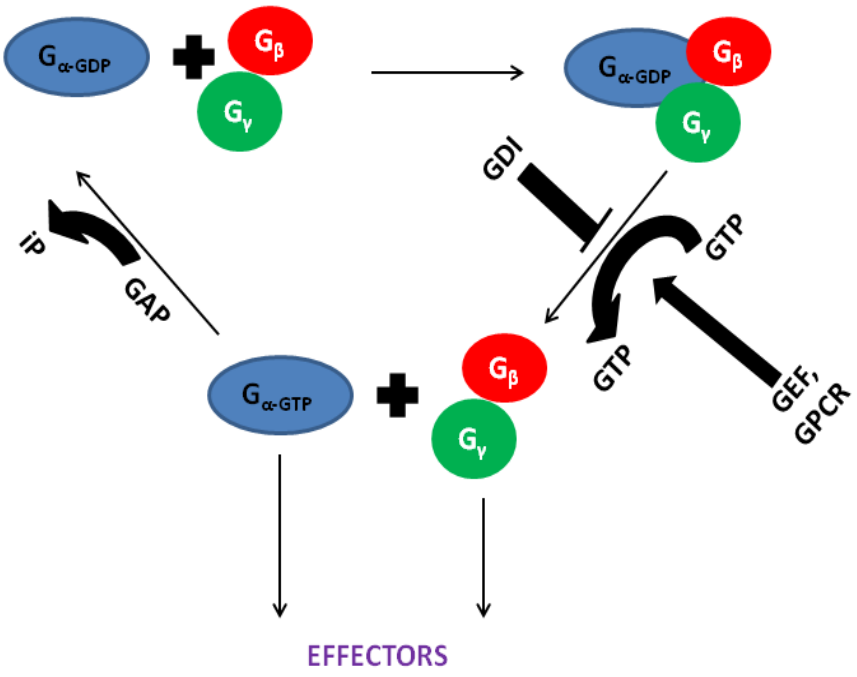

Figure 1.2: Regulation of G protein activation/inactivation cycle by Gα regulatory proteins.

Guanine nucleotide exchange factor (GEF) catalyzes the change of GDP on the inactive Gα subunit for GTP. Guanine nucleotide dissociation inhibitor (GDI), prevents this nucleotide exchange and stabilizes the Gα-GDP complex to prevent Gα signaling. GTPase activating protein (GAP), enhances the hydrolysis of GTP by Gα subunits (95).

Family	Subtypes	Effectors	Receptors
$G\alpha_s$	$G\alpha_s$ $G\alpha_s$(L) $G\alpha_s$(XL) G_{olf}	Adenylyl Cyclase/cAMP/PKA K^+, Ca^{2+} channel Src, Hck Tubulin GTPase	β2AR α2AR Glucagon Receptor
$G\alpha_{i/o}$	$G\alpha_{o1}$, $G\alpha_{o2}$ $G\alpha_{i1–i3}$ $G\alpha_z$ $G\alpha_{t1/2}$ $G\alpha_{gust}$	Adenylyl Cyclase/cAMP/PKA MAPK (βγ subunits) Tubulin GTPase Src, Hck (βγ subunits) K^+, Ca^{2+} channels	M2 muscarinic Receptor LPA Receptor β2AR
$G\alpha_q$	$G\alpha q$ $G\alpha_{11, 14-16}$	PLCβ, PIP2 hydrolysis, Ca^{2+} mobilization (internal stores), PKC p63 Rho GEF Brutons Tyr Kinase K^+ channels	Ang II R PAR-2 NK1R LPA receptor
$G\alpha_{12/13}$	$G\alpha_{12,13}$	PLD NHE3, iNOS, HSP90 RhoGEFs PKA via AKAP	PAR-1
$G_{\beta\gamma}$		Gα subunits K^+ channels, voltage gated Ca^{2+}, Cl^- and Na^+ channels PLCβ, PLCε Rho GEFs: pRex1, p114 Rho GEF	

Figure 1.3: Effectors of heterotrimeric G proteins:

A variety of extracellular ligands can activate heterotrimeric G protein signaling. The Gα subunit is subdivided into 4 classes: $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{12/13}$ and each of these subunits can activate different downstream effectors following dissociation from the Gβγ heterodimer. The Gβγ dimer can also activate a number of different second messengers (96).

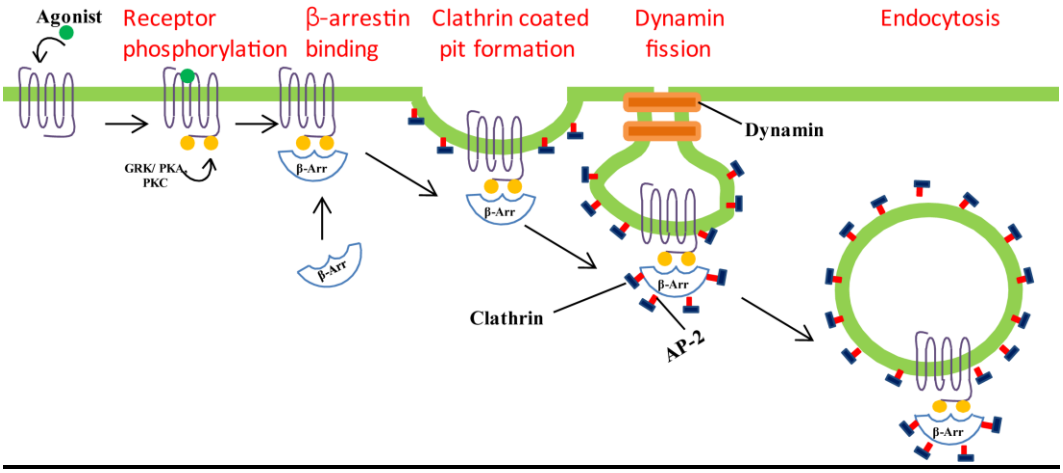

Figure 1.4: β-arrestin 1/2 are cytosolic scaffolds for terminating G protein signaling.

Agonist activated receptors are phosphorylated at multiple residues in the C-terminal tails or the third intracellular loop by second messenger kinases or G – protein coupled receptor kinases (GRKs) to mediate heterologous and homologous desensitization respectively. This leads to β-arrestin recruitment and binding to the receptor. This terminates signaling by the G protein pathway. β-arrestin also recruits AP-2 and clathrin for sequestration of activated GPCRs from the membrane (97).

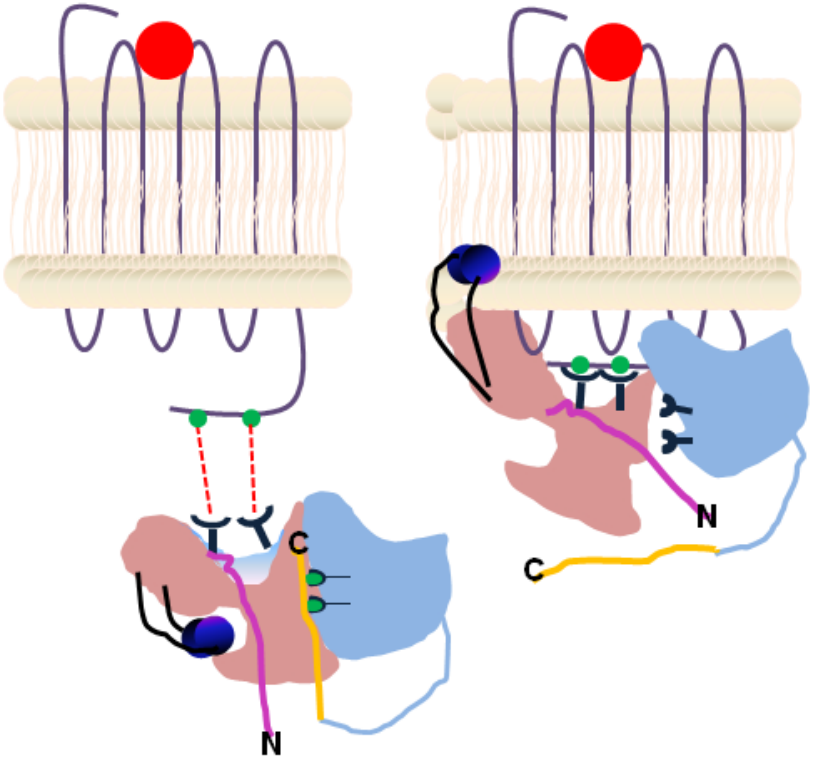

Figure 1.5: Model for receptor-β arrestin interaction: N and C terminal domains of β-arrestin have been shown in pink and blue respectively. The polar core is at the interface of the two domains. The inactive β-arrestin structure is stabilized by opposite charge interactions in the polar core area (navy-green). Hydrophobic C-tail is in yellow and the N-terminal β-strand I is in magenta. Positive and negative charges have been shown in nay and green respectively. Agonist bound receptor is in purple (98).

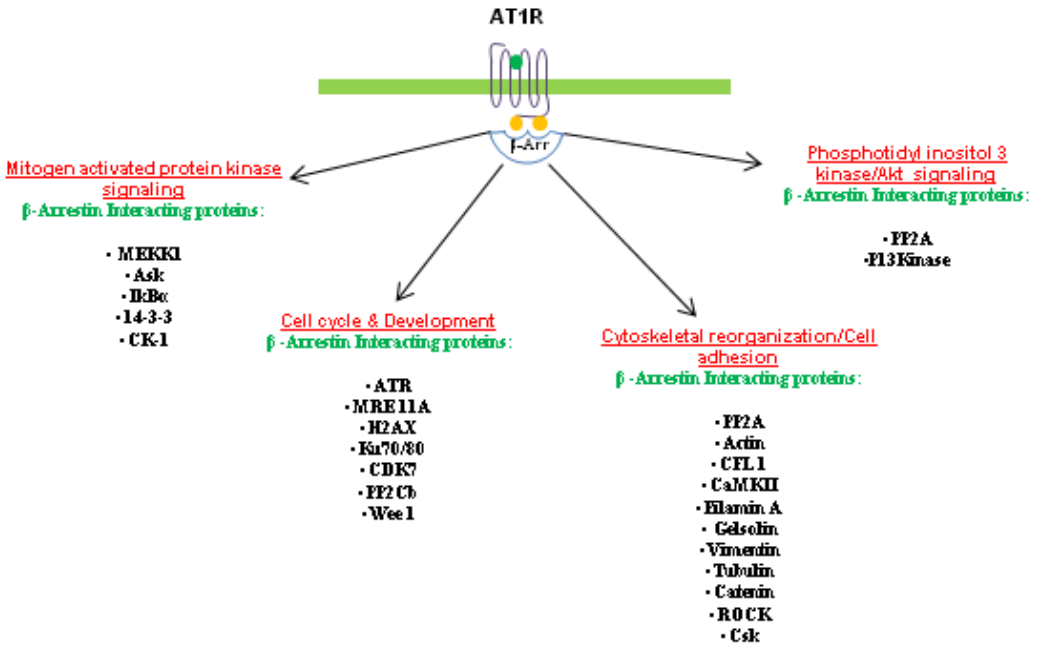

Figure 1.6: β-arrestins play non-classical roles as signal transducers

Downstream of Angiotensin receptor IA, β-arrestins have been shown to activate a variety of signaling cascades, which mediate MAPK, PI3K/Akt signaling, cell adhesion, reorganization of the cell cytoskeleton as well as cell cycle regulation (99).

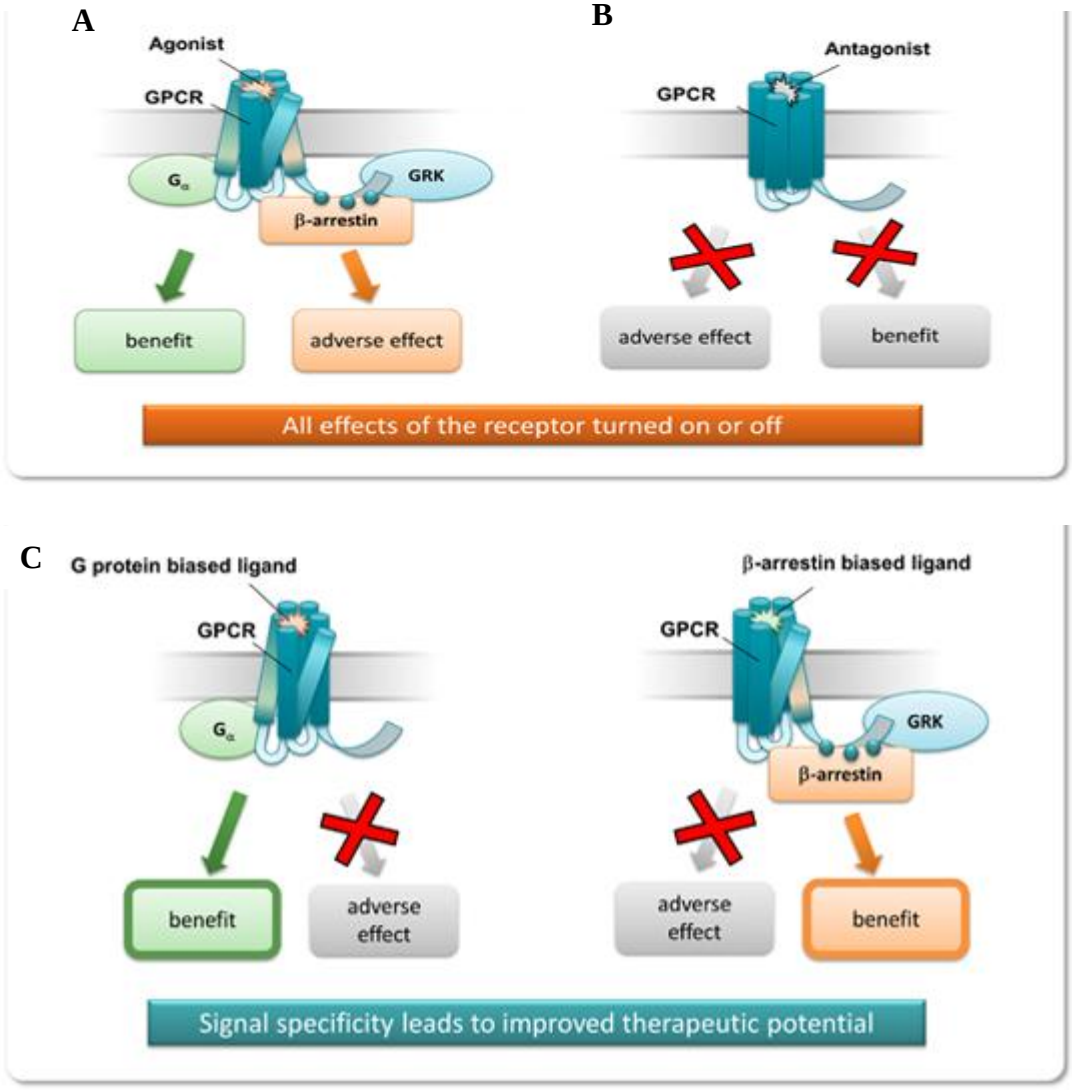

Figure 1.7: Biased signaling by GPCRs: (A) Conventional agonists of GPCRs trigger signaling by, both the signaling arms known to operate downstream of the 7TMRs, i.e., the G protein and the non-canonical β-arrestin signaling arm. (B) Similarly, antagonists block the receptor's signaling network entirely. (C) Biased ligands on the other hand target inactivation of a specific signaling arm, while allowing the same receptor engage in signaling by the alternate pathway.

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CHAPTER 2:

Differences in β-arrestin dependent signaling by PAR2 and NK1R are directed by the C-terminal tails

ABSTRACT

Protease activated receptor 2 (PAR2) and Neurokinin 1 receptor (NK1R) are G protein coupled receptors (GPCRs), which signal by the classic $G_{\alpha q}$ pathway to increase cytosolic Ca^{2+} levels and activate the second messenger protein kinase C (PKC). Both these receptors are desensitized by the binding of cytosolic scaffolding molecules β-arrestin 1 and 2, to the C-terminal tails. β-arrestin 1/2 can also mediate sequestration of these receptors by packaging them in endosomal vesicles. Apart from terminating signaling by the classic G protein pathway, β-arrestin 1/2 can also scaffold cytosolic signaling molecules, to trigger non canonical β-arrestin 1/2 dependent signaling cascades. While both PAR2 and NK1R activate Extracellular signal Regulated Kinase 1/2 (ERK1/2) through β-arrestin MAPK scaffolding complexes, PAR2 can exclusively activate the actin severing protein, cofilin. We determined that PAR2 recruits both β-arrestin 1/2, with equal affinity and more rapidly than NK1R. NK1R recruits β-arrestin2 significantly faster than β-arrestin1. Using wild type and chimeric receptors, we showed that the rate of desensitization, internalization, subcellular localization post endocytosis, mechanism of ERK1/2 activation as well as physiological responses of cell migration and proliferation depends on nature of interaction of β-arrestins with the C-termini of the respective receptor.

INTRODUCTION

PAR2 and NK1R are GPCRs, which signal by the Gαq pathway following receptor activation (1,2). However the receptors differ the mechanism of activation. NK1R is activated by the mechanism, which activates many other receptors, i.e., the reversible binding of a diffusible agonist, Substance P (SP) to the cytosolic face of the receptor. PAR2 on the other hand is activated by irreversible cleavage of the N-terminus by serine proteases, to generate a tethered ligand. Following tryptic cleavage the unmasked ligand can fold over and interact with the second extracellular loop of the receptor to activate it (3). Following receptor activation, both the receptors signal by Gαq pathway (2,4). In addition to Gαq, PAR2 is also known to signal by $G\alpha_{12/13}$ to activate the small GTPase Rho (5).

A clear understanding of the mechanisms regulating PAR2 desensitization is important, as it is activated by irreversible receptor cleavage (6). Unlike other receptors, which are activated by ligand binding, PAR2 is expected to be constitutively active, once activated. Following the initial activation, any change in the concentration of the activating proteases in the media, will not affect the cellular responses generated by the receptor.

One of the major mechanisms that prevents constitutive signaling and shuts off PAR2 is the internalization process. NK1R following activation is

phosphorylated at serine/threonine residues in the C-tail and internalized by scaffolding of clathrin by β-arrestin1/2. Following internalization, the receptor is recycled back to the membrane (7-9). PAR2 on the other is trafficked to the lysosomes for degradation. PAR2 expressing cells have an intracellular reservoir of PAR2 in the golgi vesicles. Following degradation of protease cleaved receptor, new PAR2 is replenished at the membrane through vesicular trafficking from the trans-golgi network (10).

Apart from the post endocytotic trafficking, PAR2 and NK1R also differ in their signaling pathways [Figure 2.1]. Both the receptors can activate ERK 1/2 downstream of the Gq pathway. Gq activates membrane bound enzyme Phospholipase C β (PLC β), which breaks down the phospholipid phosphotidyl inositol-4,5-biphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular organelles. This cytosolic rise in Ca^{2+} along with DAG activates PKC(11,12). Using pharmacological inhibitors of PKC, it has been demonstrated that, ERK1/2 phosphorylaiton is suppressed by greater than 50% in KNRK cell lines downstream of PAR2. However it is unknown whether PKC directly phosphorylates ERK1/2 or the upstream activator Raf-1. Gq activated ERK downstream of PAR2, is translocated to the nucleus for proliferation (13). In case of NK1R, Gq mediated MAPK kinase activation requires activation of intermediates like PYK-1, Src, Shc, Ras, Raf-1, Mek-1 and ERK1/2. Apart from

the classic Gq pathway, β-arrestins can also activate MAPK pathways downstream of the two 7TMRs independently of the canonical pathways (14).

PAR2 is uncoupled from the G protein pathway and internalized by binding of β-arrestin to the C-terminal. In addition β-arrestin can scaffold components of the MAPK pathway to activate ERK1/2. Confocal microscopy showed that, β-arrestin colocalized with Raf-1 following trypsin stimulation of KNRK cells expressing PAR2. Using gel filtration chromatography and co-immunoprecipitation, it has been shown that following agonist stimulation, PAR2, β-arrestin-1, Raf-1 Mek-1 and pERK coeluted as a complex in a single fraction. β-arrestin$^{318-419}$, a dominant negative mutant of β-arrestin-1 incapable of receptor internalization also forms a similar complex. However, the ERK1/2 found in the β-arrestin$^{318-419}$ was found to be inactive, thus indicating that receptor internalization is a crucial step in β-arrestin dependent activation of ERK1/2. The activated ERK1/2 downstream of this pathway is restricted to the cytosol, where it promotes rearrangement of actin cytoskeleton, to generate membrane protrusions for cell migration (13).

Similar experiments showed that NK1R is also capable of activating ERK 1/2, by the β-arrestin pathway. However, the components of the scaffolding complex were different from the PAR2-β-arrestin complex. SP activated NK1R forms a complex with β-arrestin, Src, Ras and pERK. However, Raf-1 was absent

in this complex. It believed that the, differences in the components of the scaffolding complex determines the stability of the activated pERK. ERK1/2 activated by the β-arrestin dependent pathway is translocated to the nucleus where it promotes proliferation. The NK1R/β-arrestin/MAPK scaffold is unstable and could be co-eluted together in a single fraction only if the lysates were crosslinked with DSP(14). This lowered stability of the NK1R/ β-arrestin/MAPK scaffold might me a possible reason for nuclear translocation of pERK1/2 in this case.

Thus, PAR2 and NK1R differ in their post endocytotic fates as well β-arrestin dependent ERK activation. This can be attributed to the nature of interaction of β-arrestin 1/2 molecules with the receptor. β-arrestin 1/2 are known to bind to receptor C-tails as well as the third intracellular loop of the receptor in the cytoplasm. The nature of β-arrestin interaction with the GPCR is diverse. In certain GPCRs, agonist stimulation leads to β-arrestin binding to the cytosolic C terminal tail. The third intracellular loop of the receptors can also serve as a binding interface and in some instances both the intracellular loops as well as the C-terminal tails are essential for β-arrestin binding.

The Thyrotropin-Releasing Hormone receptor (TRH) binds β-arrestin 1/2 using specific phosphorylated residues in the C-tail. These phosphosites are clustered into proximal and distal phosphosites. β-arrestin 1/2 binding to either of these locations leads to enhanced agonist affinity. However, β-arrestin binding to

the proximal cluster facilitates receptor desensitization and internalization (15). Using site directed mutagenesis, β-arrestin 1/2 binding sites have been identified in the third intracellular loop of the α_{2B}-adrenergic receptor. Although, mutation of these residues does not affect ERK activation, there is complete abrogation of agonist mediated receptor internalization (16). In case of the D1-dopamine receptor, the β-arrestin 1/2 binding interface lies in the third intracellular loop (IL3) of the receptor. Phosphorylation of serine/threonine residues in both the receptor C-tail as well as the IL3 is crucial for β-arrestin 1/2 binding. The C-terminal tail masks the β-arrestin 1/2 binding sites in the IL3, in the inactive conformation. Phosphorylation of residues in the C-tail and the IL3 generates repulsion due to like charges, which displaces the C-tail, allowing the β-arrestin molecule to bind to IL3 (17).

In addition to the presence of binding sites, the C-terminal tails of GPCRs also harbor endocytotic, sorting and recycling motifs (18). C-terminal tails of GPCRs are also the crucial sites for post-translational modifications like ubiquitination, palmitoylation and nitrosylation, which regulate various facets of signaling (18-23).

Keeping these facts in mind, we can claim that C-terminal tails of GPCRs are important structural determinants, which govern β-arrestin binding to the activated receptors and thereby regulate receptor desensitization, internalization

as well as some of the unique G protein independent signaling pathways. Since, PAR2 and NK1R differ vastly in their intracellular sorting pathways, mechanism of β-arrestin dependent ERK activation and the subsequent ERK mediated cellular outcomes, we hypothesized that these differences can be attributed to the nature of interaction of β-arrestin 1/2 with the C-tail.

From a physiological standpoint, investigating the similarities and differences between PAR2 and NK1R is important. A previous study by Steinhoff *et al.,* has reported that tryptase mediated activation PAR2 in primary spinal afferent neurons, leads to expression of SubP, which in turn transactivates NK1R on endothelial cells. This leads to pro-inflammatory effects in neurons (24). Signaling by PAR2 and NK1R are therefore intertwined to generate inflammation in human disease conditions. Thus, a better understanding of the molecular mechanisms that govern signaling by these two GPCRs is essential for designing anti-inflammatory drugs for therapeutic purposes.

MATERIALS & METHODS

Materials

Unless mentioned all reagents were purchased form Sigma. The following primary antibodies were used for western blotting or immunostaining: Rabbit anti phospho-cofilin (Cell Signaling); mouse anti total-cofilin (BD Transduction laboratories); rabbit anti phospho-ERK1/2 (Cell Signaling); mouse anti total-ERK1/2 (Cell signaling); mouse monoclonal antibody to EEA-1 (BD transduction laboratories); rabbit anti LAMP1 (Santa Cruz Biotechnology). AF546 tagged secondary antibody to mouse and rabbit were obtained from Invitrogen. IRDye®680 and IRDye®800 tagged secondary antibodies (Rockland) were used for western blotting and on cell western assays, to enable visualization using the LICOR odyssey imaging system. 2-furoyl-LIGRLO-NH2, [Sar-9, Met(O2)-11]-Substance P were purchased from Tocris. Renilla Luciferase tagged β-arrestin 1 and 2 constructs were obtained as gifts from Dr. JoAnn Trejo and Dr. Michel Bouvier (University of Montreal).

Cell culture and transfection

Human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) cell lines were grown in 10% (v/v) fetal calf serum (FCS) supplemented Dulbecco's modified Eagle's media (DMEM) and DMEM F-12 respectively and maintained at 37°C and 5% CO_2. The cells were passaged using Cell stripper solution (Cellgro). For stable transfection of plasmids in CHO cells, Lipofectamine and

Plus reagent (Invitrogen) was used, using the manufacturer's protocols and subjected to G418 selection. For BRET, phospho-ERK1/2, cell migration and cofilin dephosphorylation assays, transient transfections were carried out in 80% confluent HEK 293 or CHO cells using FuGene6 (Roche) following the manufacturer's protocols.

Bioluminescence Resonance Energy Transfer (BRET)

β-arrestin1/2-RLuc and eYFP tagged receptor constructs were transiently co-expressed in HEK-293 cells. 24 hours after transfection, the cells were distributed in poly-lysine coated 96 well plates (white bottomed). 48 hours post transfection, the cells were treated with appropriate concentrations of 2fAP or Sub P and 5uM coelenterazine. Readings were taken 15 minutes after agonist stimulation in case of dose response curves. When required, pharmacological inhibitors were added at appropriate concentrations and the cells were incubated at 37°C, prior to addition of 2fAP. Light emission was detected (460–500 nm for RLuc and 510–550 nm for YFP) using a TRISTAR LB941 multilabel plate reader from Berthold Technologies. BRET signal was calculated as the ratio of the light emitted by eYFP and the light emitted by luciferase. As negative control, cells transfected with the luciferase construct alone were used to determine the background. Half lives ($t_{1/2}$) of the kinetics reactions were determined from 5 separate experiments.

Immunofluorescence and Confocal microscopy

For the receptor internalization assays, 3×10^4 CHO cells stably expressing PAR2-GFP, NK1R-GFP, PAR2-NK1R-GFP and NK1R-PAR2-GFP were cultured

overnight on collagen coated coverslips. 24 hours post transfection media was changed to serum free DMEM. After incubation on ice, the cells were treated with appropriate agonists i.e., 1uM 2fAP or 100nM SubP for 0-120 minutes. Cells were treated with 2mM leupeptin, prior to agonist addition for colocalization experiments with LAMP-1. Following agonist treatment, the cells were washed with ice-cold 1X PBS, fixed with 4% paraformaldehyde and blocked using 5% GB. Immunostaining was carried out using the following concentration of antibodies: Mouse anti FLAG (1:250), Rabbit ant pERK 1/2 (1:250), Mouse anti t-ERK1/2 (1:250), Mouse anti EEA-1 (1:250), Rabbit anti LAMP-1 (1:250) and TOPORO-3 (1:1000). The coverslips were mounted using Vectasheild. Images were viewed using the 63X and 100X objectives of Zeiss LSM 510 confocal microscope.

Western blotting

HEK 293 cells were transiently transfected with tagged PAR2 or NK1R. 36 hours post transfection, the cells were serum starved overnight. When required the cells were treated with appropriate concentration of inhibitors or vehicle for 10 mins. Following activation with 1uM 2fAP or 100nM Substance P for 0-60 minutes, the cells were washed with 1X PBS and treated with cofilin lysis buffer (phosphate buffered saline pH 7.6, supplemented with 10mM NaF, 2mM Na_3VO_4, 1mM EGTA, 1% Triton-X100 and protease inhibitors). 30ug of the cleared protein lysates were loaded into the wells of 15% SDS-PAGE gel. Western blotting was carried out by, transferring proteins onto PVDF-FL membrane (Millipore) and

blocking in 1% fish gelatin in TBS. All primary antibodies were used at a concentration of 1:1000. The blots were scanned using LICOR Odyssey imaging system. The bands were quantified using the LICOR software. Western blot images were processed by Adobe Photoshop CS3 and placed in Adobe IllustratorCS3.

Cell Migration assay

CHO or HEK-293 cells were transiently transfected with PAR2, NK1R, PAR2NK1R or NK1RPAR2. 48 hours post transfection, $2x10^4$ cells were transferred to collagen coated 5μm (6.5mm diameter) transwell supports and allowed to attach for 2 hours at 37°C. Following addition of 10μM 2fAP or 100nM Sar-Met-SP to the lower chambers of the permeable supports, the cells were incubated at 37°C for 4 hours. The total number of cells migrated to the bottom of the filter were stained with crystal violet and quantified by under 20X objective of Nikon phase contrast microscope.

Proliferation assay

CHO cells expressing GFP-tagged PAR2, NK1R, PAR2-NK1R or NK1RPAR2 chimera were cultured in 35mm dishes and allowed to attach for 2 hours. Following overnight serum starvation, they were treated with 2fAP, SubP or serum. Following detachment, cells were incubated with fluorescent dye propidium iodide (PI) and resuspended in FACS buffer. Number of cells per ml was quantified using Beckman flow cytometer.

RESULTS

To determine whether the differences in β-arrestin 1/2 dependent signaling by PAR2 and NK1R is dependent on the nature of β-arrestin1/2 interaction with the receptor C-tail, we used Bioluminescence Resonance Energy Transfer (BRET) to determine protein-protein interaction in real time and in live cells. Results of BRET dose response assays with 2-furoyl-LIGRL (2fAP) and SP [Figure 2.1 (A) & (B)] showed that there is dose dependent incremental recruitment of β-arrestin 1 and 2 to PAR2 and NK1R. Next, β-arrestin 1/2 recruitment, to the C-terminal tails of the two GPCRs was monitored over a period of 20 minutes using BRET kinetics assays. Results showed that while PAR2 recruited both β-arrestin 1/2 with equal efficacies, NK1R preferentially recruited β-arrestin-2 over β-arrestin 1. On computing the rates of the reactions we observed that the rate of β-arrestin 1/2, recruitment to NK1R were nearly 2 fold slower than that for PAR2 [Figure 2.2 (C) & (D)].

We hypothesized that this difference in recruitment rate maybe either due to the differences in binding affinities of β-arrestin 1/2 towards the C-terminal tail of the receptor or due a difference in the orientation of the donor (Luciferase) and acceptor (eYFP) during the binding event. To test this hypothesis BRET titration assays were designed. For these assays we co-expressed varying levels of Rluc-β-arrestin 1/2 and receptor-eYFP. Thus, we had different pools of cells with

increasing acceptor to donor ratio. If the interaction between the two proteins of interest is specific, i.e., not due to random collision, we expect to see a hyperbolic rise in BRET signal with increasing acceptor to donor ratio. The plateau phase is observed, as all of the β-arrestin 1/2 binding sites on PAR2 or NK1R C-tails have been completely saturated. This saturation phase is labeled $BRET_{max}$. Any difference in $BRET_{max}$ values between two different sets of interacting proteins, indicate a change in the orientation of the fusion proteins. The acceptor to donor ratio at which half maximal BRET signal is observed is called $BRET_{50}$. $BRET_{50}$ is similar to K_D or dissociation constant, which indicates binding affinity of two proteins. A lower $BRET_{50}$ indicates, a higher binding between the interacting proteins.

BRET titration assay showed that $BRET_{50}$ value for the NK1R/β-arrestin combination was significantly higher by nearly 2 fold when compared to the PAR2/β-arrestin-1 and 2.7 fold for PAR2/β-arrestin-2. This indicates that the binding affinity of the β-arrestin-1/2 molecules for the PAR2 C-terminal is significantly reduced. Furthermore, the affinity of β-arrestin-2 to NK1R was significantly higher than β-arrestin-1, as indicated by the $BRET_{50}$ value [Figure 2.2 (E) & Table 1]. In addition, the $BRET_{max}$ values for the NK1R/β-arrestin-1 interacting combination was significantly different from the other receptor/β-arrestin combinations tested in the assay [Figure 2.2 (E) & Table 1]. This

indicates a difference in the conformation with which β-arrestin-1 and 2 bind to the C-tail of NK1R.

In order to determine whether the C-terminal tails of the two GPCRs are sufficient to generate these differences in β-arrestin-1/2 recruitment and binding we generated chimeric receptors by swapping the C-tails of the two receptors. We cloned the C-terminal of PAR2 to the seventh transmembrane domain of NK1R to generate the PAR2-NK1R chimera and did the vice-versa to make a PAR2-NK1R receptor. The cDNA was then subcloned in the eGFP-N1 or eYFP-N1 vector for use in confocal microscopy and BRET assays [Figure 2.3].

Immunofluorescence microscopy was first carried out to determine whether the chimeric receptors colocalized with β-arrestin-1/2 following agonist treatment, similar to the wild type receptors. In untreated conditions, we observed that the wild type and chimeric receptors were expressed at the membrane and β-arrestin-1 and 2 were dispersed throughout the cytoplasm. Within 5 minutes of agonist stimulation, the chimeric receptors colocalize with β-arrestin-1/2 at the membrane, similar to the wild type receptors. By 30 minutes all four receptors have been internalized and β-arrestin-1/2 still continue to remain co-localized with the receptors in the cytoplasm, as indicated by a number of intra-eellular vesicles [Figure 2.5].

Although confocal microscopy gave us a visual indication of β-arrestin-1/2 association with the wild type and chimeric receptors as well as cellular compartments at which colocalization sets in, in order to determine subtle variations in β-arrestin-1/2 recruitment rates, we carried out BRET assays using the C-terminal chimeric receptors. Results of the BRET assay showed that the PAR2 receptor with the NK1R C tail on stimulation with 2fAP, no longer recruits both β-arrestin-1/2 with similar efficacy. It shows preferential recruitment of β-arrestin-2 over β-arrestin-1, like the wild type NK1R receptor. On the contrary, the NK1R-PAR2 chimera, following SubP stimulation, brings about robust binding to both β-arrestin-1/2, similar to wild type PAR2 [Figure 2.4].

Once we determined that the C-terminal tail alone was sufficient to direct the differences in β-arrestin-1/2 recruitment and binding to the C-tail of PAR2 and NK1R, we went on to determine whether any of the β-arrestin mediated GPCR regulatory functions like receptor desensitization, internalization or ERK1/2 signaling is affected by switching the C-tails.

Whole cell Ca^{2+} mobilization assays were used to determine signaling by Gq pathway. Since both NK1R and PAR2 signal by the $G_{\alpha q}$ pathway to generate Ca^{2+} as a second messenger, rise in cytosolic levels of Ca^{2+} can be used as, a readout for $G_{\alpha q}$ signaling. Following PAR2 stimulation, we observed a rapid rise in cytosolic Ca^{2+} levels, which immediately drops to baseline in 100 seconds. This

shortened duration of Ca^{2+} rise is also observed in NK1R-PAR2. NK1R on the other hand shows prolonged Ca^{2+} signaling. This sustained Ca^{2+} rise is also demonstrated by activated PAR2-NK1R. These differences in Ca^{2+} signaling duration can be correlated to the rate of β-arrestin-1/2 recruitment to the respective receptors. Since, PAR2 and NK1R-PAR2 both recruit β-arrestin-1/2 at significantly faster rates than NK1R and PAR2-NK1R, they are uncoupled from the cognate G protein at earlier time points, which explains the faster desensitization (25).

We next onto determine whether the differences in β-arrestin-1/2 recruitment also affect receptor internalization. Using confocal microscopy we monitored the time points at which the wild type and chimeric receptors colocalize with the early endosomal marker EEA-1. We observed that the PAR2 and the NK1R-PAR2 receptors start to colocalize with EEA-1 as early as 5 minutes of agonist stimulation. The NK1R and PAR2-NK1R chimera showed colocalization after 15 minutes of agonist stimulation. This difference in receptor internalization can also be attributed to the rate of β-arrestin-1/2 recruitment. As β-arrestin-1/2 can scaffold clathrin and Adaptor Protein-2 (AP-2) to facilitate GPCR internalization, rate of β-arrestin-1/2 recruitment to the receptors is a crucial determinant for endocytosis. Since, PAR2 and NK1R-PAR2 recruit both β-arrestins at a significantly higher rate than NK1R and PAR2-NK1R, internalization of the receptors is initiated at earlier time points [Figure 2.6].

The chimeric receptors followed post endocytotic sorting fates similar to their C-terminal parent. PAR2 following internalization is channeled to lysosomes for complete degradation, NK1R on the other hand is trafficked to the membrane on recycling vesicles. We observed colocalization of the chimeric receptors with the lysosomal marker LAMP-1. While NK1R-PAR2 colocalized with LAMP-1 like wild type PAR2, the PAR2-NK1R chimera did not similar to wild type NK1R. Hence, it appears that differences in β-arrestin-1/2 binding to GPCRs also affect subcellular trafficking fates [Figure 2.7].

We next went on to determine whether β-arrestin-1/2 dependent ERK1/2 [also known as p42/p44] activation and the differences in subsequent ERK1/2 mediated physiological responses, are also determined by the β-arrestin-1/2 binding to receptor C-tails. Western blot analyses showed that the chimeric receptors promoted phosphorylation of p42 and p44 following agonist stimulation, like the wild type receptors. To determine if the subcellular location of activated pERK1/2 differed in the chimeric receptors, we used immunofluorescence microscopy. While the pERK 1/2 activated by NK1R-PAR2 is restricted in the cell cytoplasm similar to PAR2, PAR2-NK1R promoted nuclear translocation of activated ERK 1/2 [Figure 2.8].

Finally, transwell migration assays and scratch assays were carried out to determine whether the ultimate physiological responses of cell were directed by

the subcellular location of activated pERK1/2. While the PAR2 chimera with the NK1R C-terminal was unable to carry out cell migration, it promoted robust cell proliferation at comparable levels as the NK1R wild type receptor or the serum positive controls. The NK1R-PAR2 chimera on the other hand triggered robust cell migration like the wild type PAR2 receptor. Thus we observed that switching the C-terminal tails of PAR2 and NK1R affects all of the β-arrestin-1/2 dependent GPCR regulatory functions, i.e., desensitization, internalization as well as β-arrestin-1/2 dependent ERK1/2 signaling pathways [Figure 2.9].

DISCUSSION

PAR2 and NK1R are $G_{\alpha q}$ coupled 7 transmembrane receptors. While PAR2 is activated by irreversible cleavage of extracellular N terminus, NK1R activation requires reversible binding of the extracellular soluble ligand Substance P. However, the desensitization of the two receptors follows a similar pattern. This is involves binding of the cytosolic scaffolding proteins β-arrestins 1/2 to the C –tail of the receptor. This generates steric hindrance to uncouple the receptor-$G_{\alpha q}$ association. β-arrestins 1/2 also binds components of the cellular endocytotic machinery to sequester the receptors from the membrane in clathrin-coated pits. Following internalization the two receptors differ in their unique β-arrestin 1/2 dependent pathways. Both the receptors lead to ERK1/2 activation by these pathways. However, the difference in the components of the β-arrestin scaffolding complex, leads to membrane retention of ERK1/2 downstream of PAR2 whereas in case of NK1R, ERK1/2 is translocated to the nucleus. We hypothesized that the, difference in β-arrestin1/2 dependent pathways in the case of the two GPCRs depend on nature of interaction of β-arrestin1/2 with the C-tail of the receptors.

In this investigation we have shown that activated NK1R demonstrates prolonged Ca^{2+} signaling compared to PAR2. Chimeric receptors, generated by switching the C-termini of the receptors shows the opposite effect, i.e., 2fAP activated PAR2NK1R has a longer duration of Ca^{2+} signaling than SP stimulated

NK1RPAR2. After comparing receptor internalization patterns, we determined that agonist activated PAR2 internalizes more rapidly than NK1R. PAR2NK1R on the other hand follows the slower internalization rates of NK1R. On the contrary NK1RPAR2 is rapidly removed from the membrane like its C-terminal parent. These properties of receptor desensitization and internalization are mediated by β-arrestin1/2 and these distinct patterns can be attributed to nature of β-arrestin association with the C-tail of the respective receptor. Both the wild type and the chimeric receptors are capable of ERK1/2 activation. However, the subcellular localization of activated phospho-ERK1/2 in the chimeric receptors follows the same trend as the C-tail parent. Thus, PAR2 and NK1R show distinct trends with regards to β-arrestin mediated desensitization as well as signaling.

Results of BRET kinetics assays show that PAR2 recruits both β-arrestin 1 and 2 at a much more rapid rate compared to NK1R. This explains the difference in the desensitization and internalization rates in case of PAR2 and NK1R. We have also shown that NK1R preferentially recruits β-arrestin -2 at a significantly faster rate than β-arrestin-1. It has been previously reported that β-arrestin-1 binds to internalized PAR2 and targets it for lysosomal degradation (10). Although it has not been determined whether β-arrestin-1 plays the same role in case of other GPCRs, it maybe possible that NK1R is not directed to lysosomes, as β-arrestin-1 association takes place at a later time point. Also the stability of the NK1R/ β-arrestin-1 complex is weak, which might lead to dissociation of β-arrestin-1.

We also looked into two β-arrestin1/2 dependent signaling pathways, i.e., the ERK1/2 and cofilin pathways. While all four of the receptors were capable of ERK1/2 activation, the subcellular location of activated ERK1/2 differed. Activation of PAR2 and NK1R-PAR2 leads to prolonged membrane retention of p-ERK1/2. On the contrary, NK1R and PAR2/NK1R translocates activated ERK1/2 to the nucleus. Furthermore, NK1R fails to promote cofilin dephosphorylation unlike PAR2. This can be explained on basis of the $BRET_{50}$ values calculated from the BRET titration curve. The $BRET_{50}$ values for PAR2-β-arrestin 1/2 are significantly lower than NK1R-β-arrestin 1/2. This means that β-arrestin-1/2 has a higher affinity for the PAR2 C-tail than NK1R. Thus, the scaffolding complex formed in case of PAR2 is more stable than in case of NK1R. This can explain why ERK1/2 activated by PAR2-β-arrestin-1/2 scaffold is held at the membrane. As the affinity between NK1R and β-arrestin-1/2 is lower the scaffolding complex possibly gets dislodged from the receptor tail and enters the nucleus.

Oakley *et al.*, had classified GPCRs based on their nature of interaction with β-arrestins as well as the stability of the receptor-β-arrestin complex. The first category shows higher binding affinity with β-arrestin-2 over β-arrestin-1 and includes β2-adrenergic receptor (β2AR). β2AR is known to have transient binding with β-arrestin 1/2. Following of β2AR endocytosis, β-arrestin dissociates from the receptor close the membrane proximal areas of the cell. The second

group, which includes V2R, binds both β-arrestins 1/2 with equal affinity and the GPCR-receptor complex is stable (26,27). The stability of the GPCR- β-arrestin-1/2 complex has been reported to regulate downstream signaling in case of the vasopressin receptor (V2R). While, wild type V2R promotes cytosolic retention of phospho-ERK 1/2, the chimeric V2R with the β2AR C-tail, translocated activated ERK 1/2 to the nucleus, to promote transcription of reporter genes from the Elk-1 promoter (28).

As described before by *Oakley et al.*, this stability of GPCR- β-arrestin complex is determined by the frequency of phosphorylated clusters of serine/threonine residues in the receptor C-tail (27). BRET assays using a double brilliant biosensor (Rluc-β arrestin-eYFP), has shown that β-arrestin molecules are highly flexible and can adopt different conformations when they bind to the same receptor, stimulated by different ligands (29). It will be interesting to see determine if β-arrestin molecules bind to different receptors with different conformations and whether these differences in the intramolecular conformations of β-arrestin also affect stability of the receptor- β-arrestin complex.

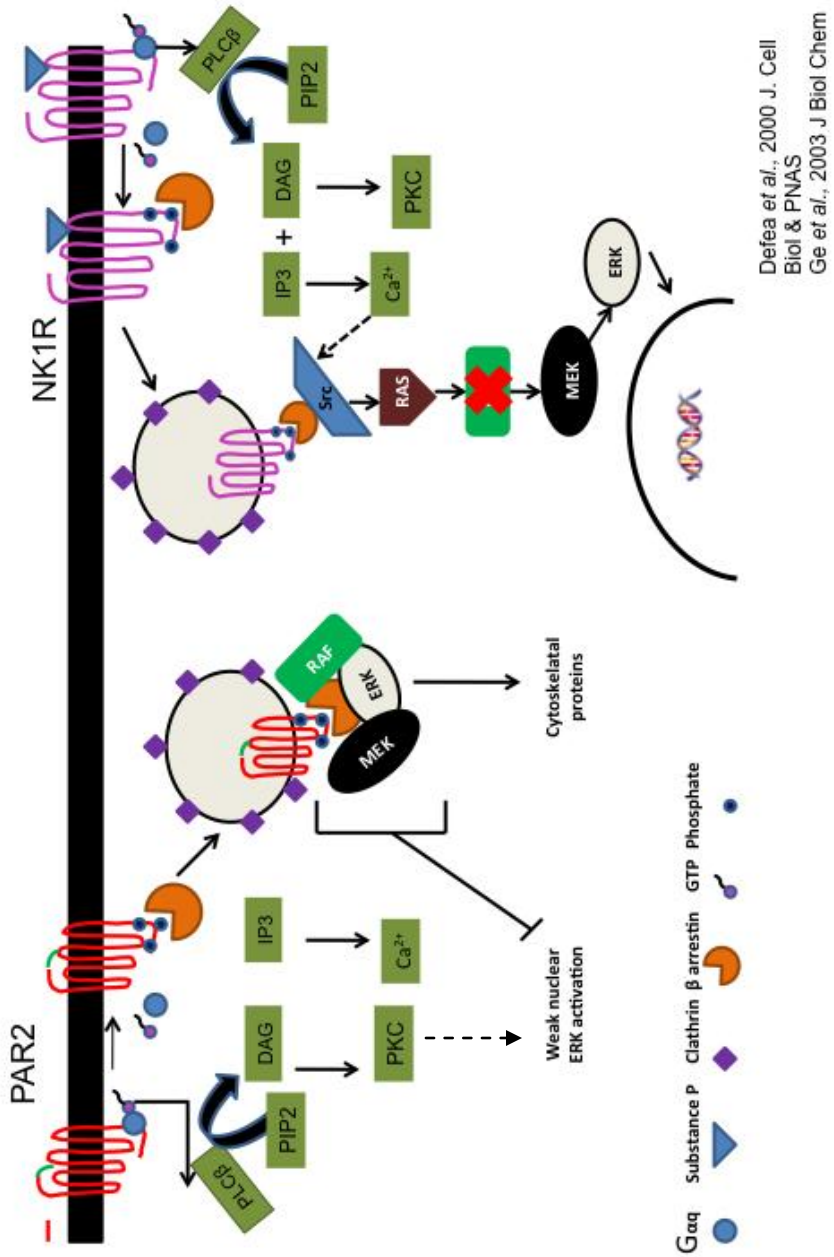
In addition, our current study also goes on provide a possible reason why PAR2 recruits β-arrestin-1/2 at faster rate than NK1R. Following activation, NK1R generates PKC through the $G_{\alpha q}$ pathway, which is expected to phosphorylate the receptor to initiate β-arrestin-1/2 association. GRK-2 is known

to actively phosphorylate NK1R in reconstituted lipid vesicles, which recruits β-arrestin-1/2 to the membrane (30). Live cell assays also show that GRK-2 and β-arrestin-2 are recruited to NK1R following SubP stimulation (31). Furthermore, BRET assays have shown that GRK-5 competes with GRK-2 for NK1R binding. It appears that GRK-5 forms a pre-associated complex with NK1R and is the key player in regulating NK1R desensitization (32). However, there are no reports of GRK phosphorylation in PAR2 desensitization. This difference may account for slower rates of β-arrestin-1/2 recruitment in case of NK1R, as it takes longer for GRK-2 and GRK-5 to establish β-arrestin-1/2 binding sites by phosphorylation.

FIGURES & LEGENDS

Figure 2.1: Comparison of signaling between PAR2 and NK1R:

The 7TMRs, PAR2 and NK1R both signal by the classic G protein pathway as well as the novel β-arrestin dependent pathway to trigger activation of ERK1/2. However, the two receptors differ in their mechanism of ERK1/2 activation. While the G protein and the β-arrestin dependent signaling arm downstream of NK1R act in concert to activate nuclear translocation of ERK1/2, the two pathways antagonize each other to restrict phosphorylated ERK1/2 at the membrane. The physiological consequence of these differences in cytosolic distribution of ERK1/2 leads to cell migration and proliferation in case of PAR2 and NK1R respectively.



Defea *et al.*, 2000 J. Cell Biol & PNAS
Ge *et al.*, 2003 J Biol Chem

Figure 2.2: PAR2 and NK1R differ in β-arrestin 1/2 recruitment rates and binding affinities: HEK-293 cells were transiently transfected with eYFP-tagged PAR2 or NK1R and Renilla luciferase tagged β-arrestin-1 or β-arrestin-2. (A-B) Concentration-response curves showing average maximal BRET responses to increasing concentrations of 2fAP or Sar-Met-SubP. (C) Cells transfected with PAR2 or NK1R were treated with 1μM 2fAP or 100nM Sar-Met-SubP respectively and BRET ratio was monitored over a period of 20 minutes. (D) Average half lives of β-arrestin1/2 recruitment to PAR2 or NK1R, estimated from 5 independent kinetic reactions and represented as mean ± SE. (E) BRET titration curve for PAR2 or NK1R and β-arrestin1/2. Cells co-transfected with a constant amount of β-arrestin1/2 and increasing amounts of PAR2 or NK1R were trated with the respective agonists for 15 mins at 37°C.

TABLE 1: Average $BRET_{50}$ and $BRET_{max}$ values estimated from 3 independent experiments.

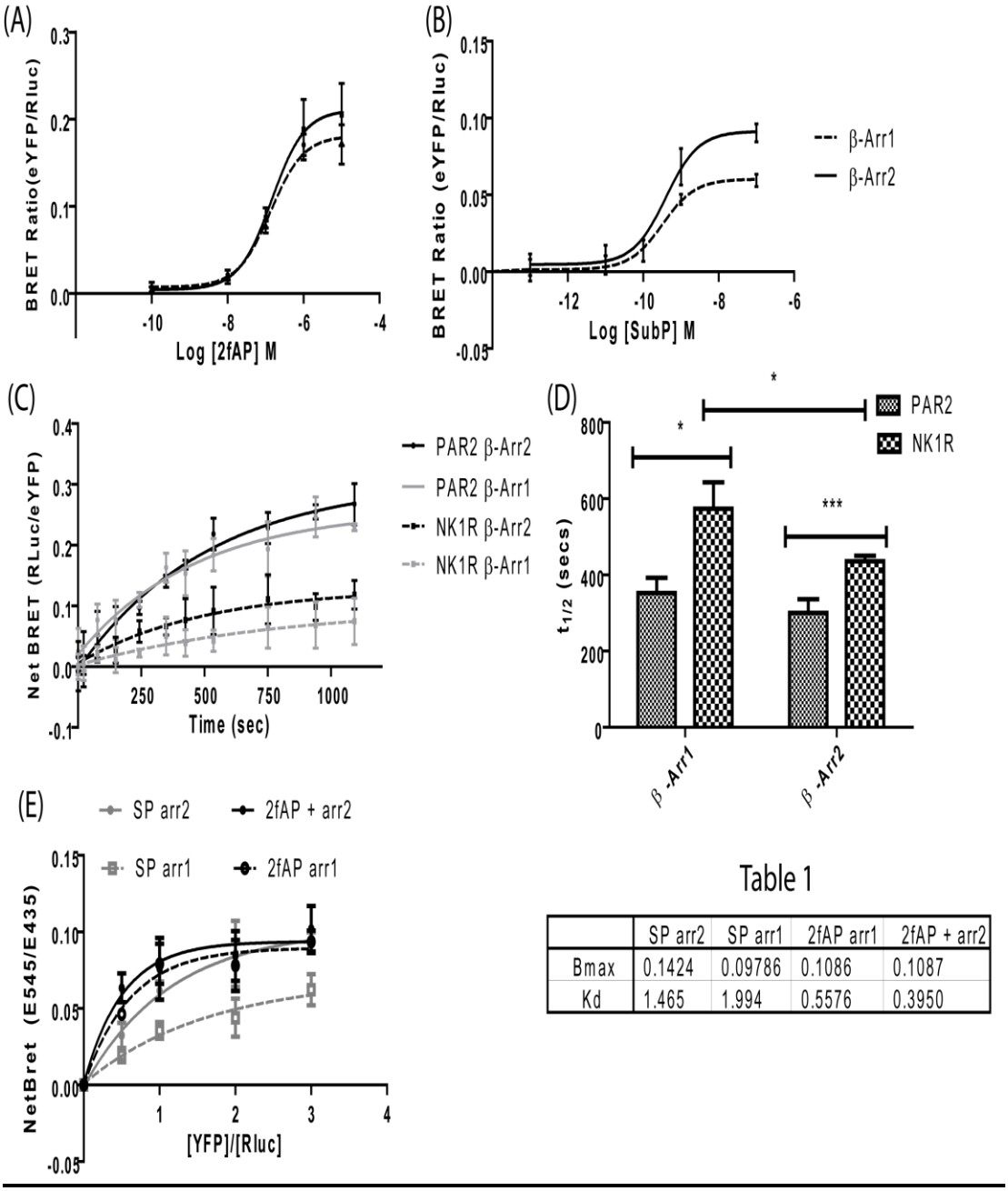


Table 1

	SP arr2	SP arr1	2fAP arr1	2fAP + arr2
Bmax	0.1424	0.09786	0.1086	0.1087
Kd	1.465	1.994	0.5576	0.3950

Figure 2.3. Generation of chimeric receptors: (A) Schematic representation of PAR2 and NK1R. Chimeric NK1RPAR2 and PAR2NK1R were generated by cloning the C-terminal tail of PAR2 to the 7^{th} transmembrane domain of NK1R and vice-versa. (B) Confocal micrographs showing membrane expression of GFP tagged PAR2, PAR2NK1R, NK1R, NK1RPAR2 in stably transfected CHO cells.

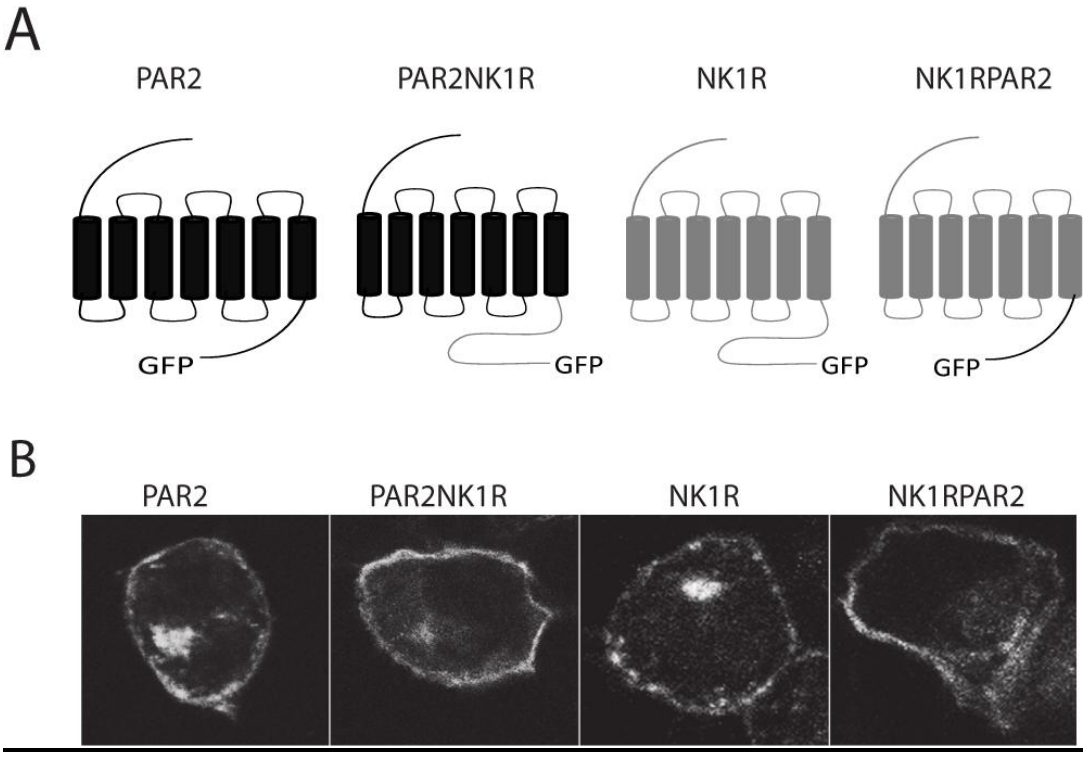

A
PAR2
PAR2NK1R
NK1R
NK1RPAR2
GFP
GFP
GFP
GFP
B
PAR2
PAR2NK1R
NK1R
NK1RPAR2

Figure 2.4. Pattern of β-arrestin1/2 recruitment of the chimeric receptors are similar to their respective N-terminal parent: HEK 293 cells transiently expressing the indicated receptors and β-arrestin1/2 were treated with 1μM 2fAP (PAR2 and PAR2NK1R) or 100nM Sar-Met-Substance P (NK1R and NK1RPAR2) and incubated at 37°C for 15 mins. Maximal BRET signal was measured following addition of 5mM of coelenterazine. ($p \leq 0.05$, n=3)

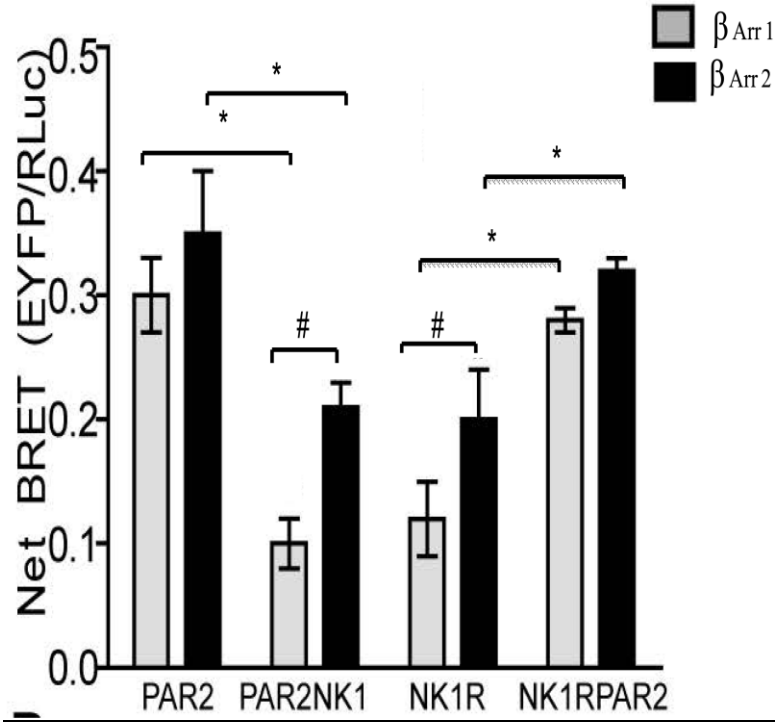

βArr 1
βArr 2
Net BRET (EYFP/RLuc)
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
*
#
#
PAR2
PAR2NK1
NK1R
NK1RPAR2

Figure 2.5: β-arrestin1/2 are recruited to agonist stimulated PAR2-NK1R and NK1R-PAR2 and remain stably associated with internalized receptors on endocytosed vesicles: CHO cells expressing GFP-tagged wild type or chimeric receptors were transiently co-transfected FLAG tagged β-arrestin1/2 followed by treatment with 1μM 2fAP or 100nM Sar-Met SP for 0-15 minutes at 37°C. The cells were immunostained for β-arrestin1/2 using anti-FLAG monoclonal antibody and observed by confocal microscopy. Arrows indicate colocalization of β-arrestin1/2 with the respective receptor. Scale bar = 10μm.

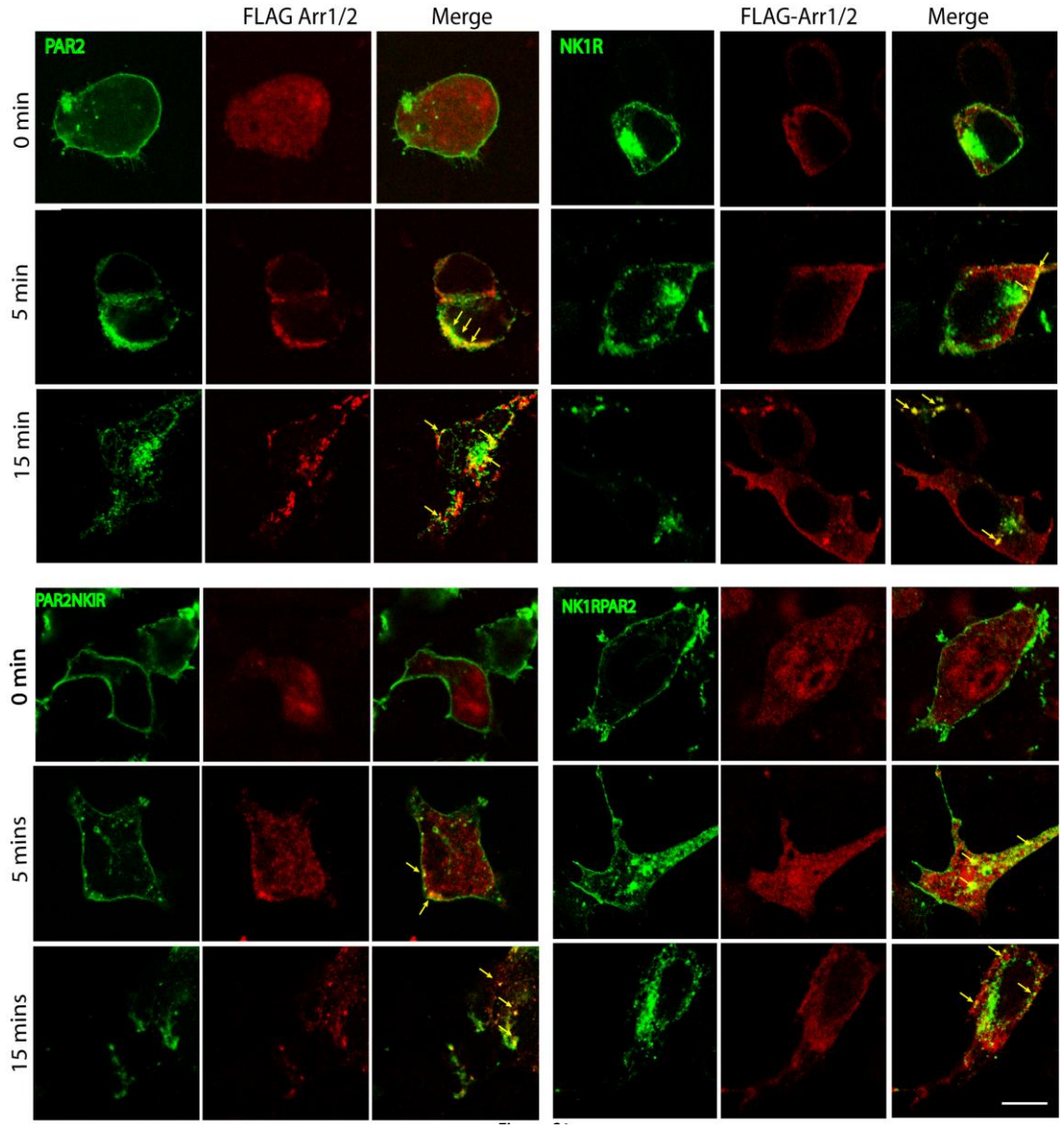

FLAG Arr1/2
Merge
FLAG-Arr1/2
Merge
PAR2
NK1R
0 min
5 min
15 min
PAR2NK1R
NK1RPAR2
0 min
5 mins
15 mins

Figure 2.6: Internalization and colocalization of PAR2NK1R and NK1RPAR2 with EEA-1 follow the same trend as wild type NK1R and PAR2 respectively. CHO cells stably expressing GFP tagged PAR2, PAR2NK1R, NK1R or NK1RPAR2 were kept on ice followed by incubation with 1μM 2fAP (PAR2 and PAR2NK1R) or 100nM Sar-Met-Substance P (NK1R and NK1RPAR2) at 37°C for the indicated time points. The cells fixed were and immunostained for endosomal marker EEA-1 for the indicated time points. Arrows show association of the respective receptor with EEA-1. Scale bar = 10μm.

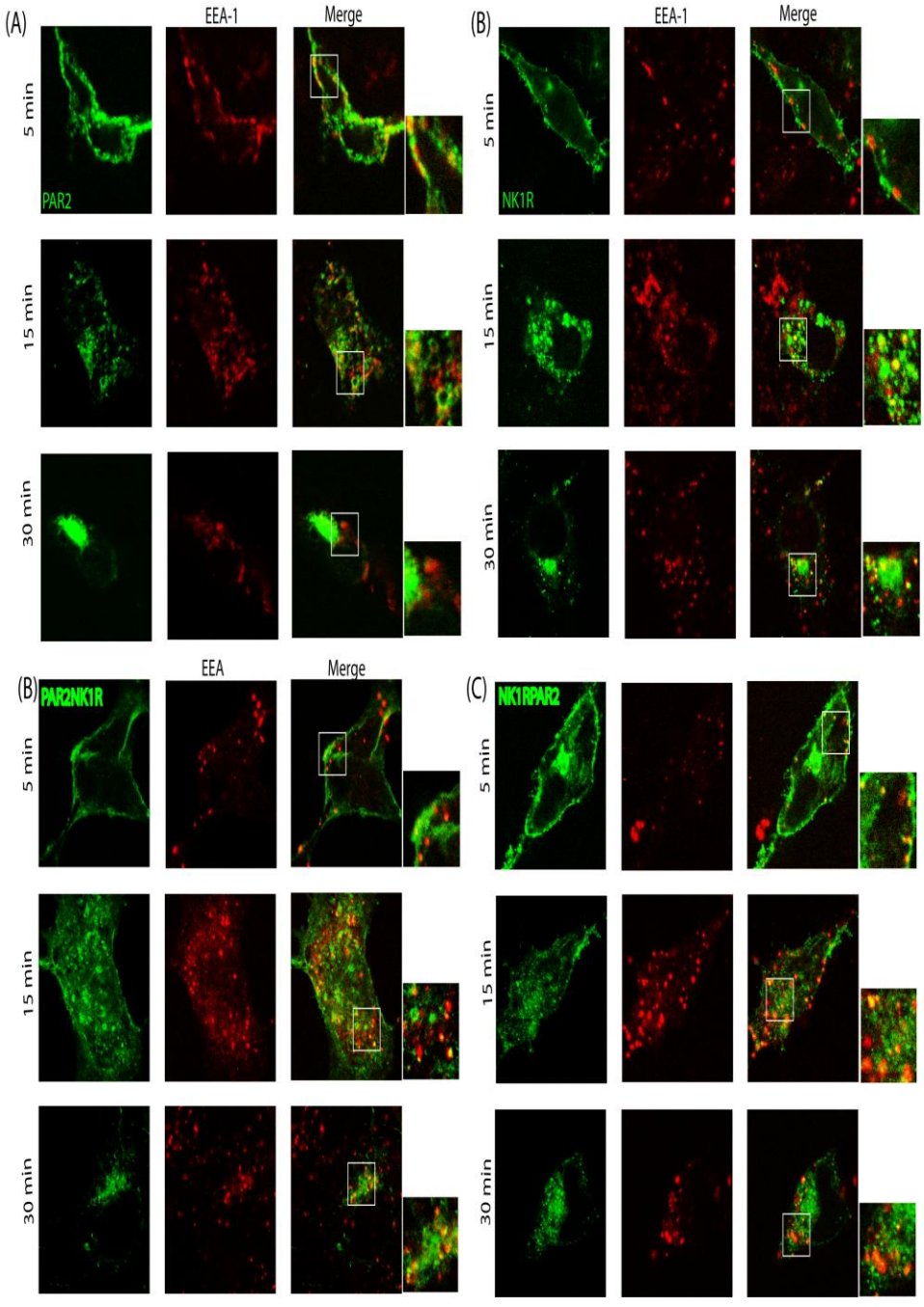

(A)
EEA-1
Merge
5 min
PAR2
15 min
30 min
(B)
EEA-1
Merge
5 min
NK1R
15 min
30 min
(B)
EEA
Merge
PAR2NK1R
5 min
15 min
30 min
(C)
NK1RPAR2
5 min
15 min
30 min

Figure 2.7: Unlike NK1RPAR2, PAR2NK1R is not sorted to lysozomes following internalization. CHO cells expressing GFP tagged receptors were treated with 1μM 2fAP or 100nM Sar-Met SP for 1 hour at 37°C. The cells were fixed and immunostained for the lysozomal marker LAMP1. Scale bar =10μm.

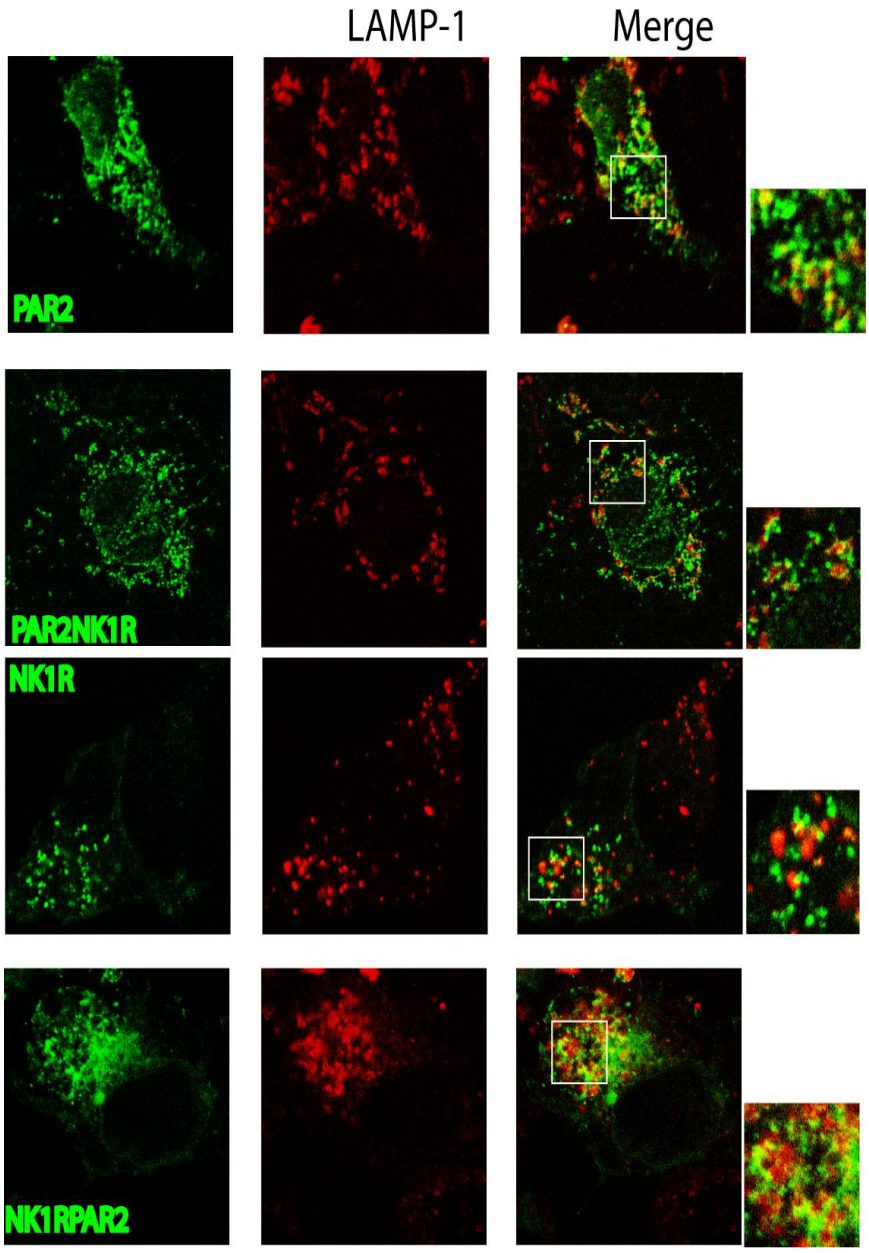
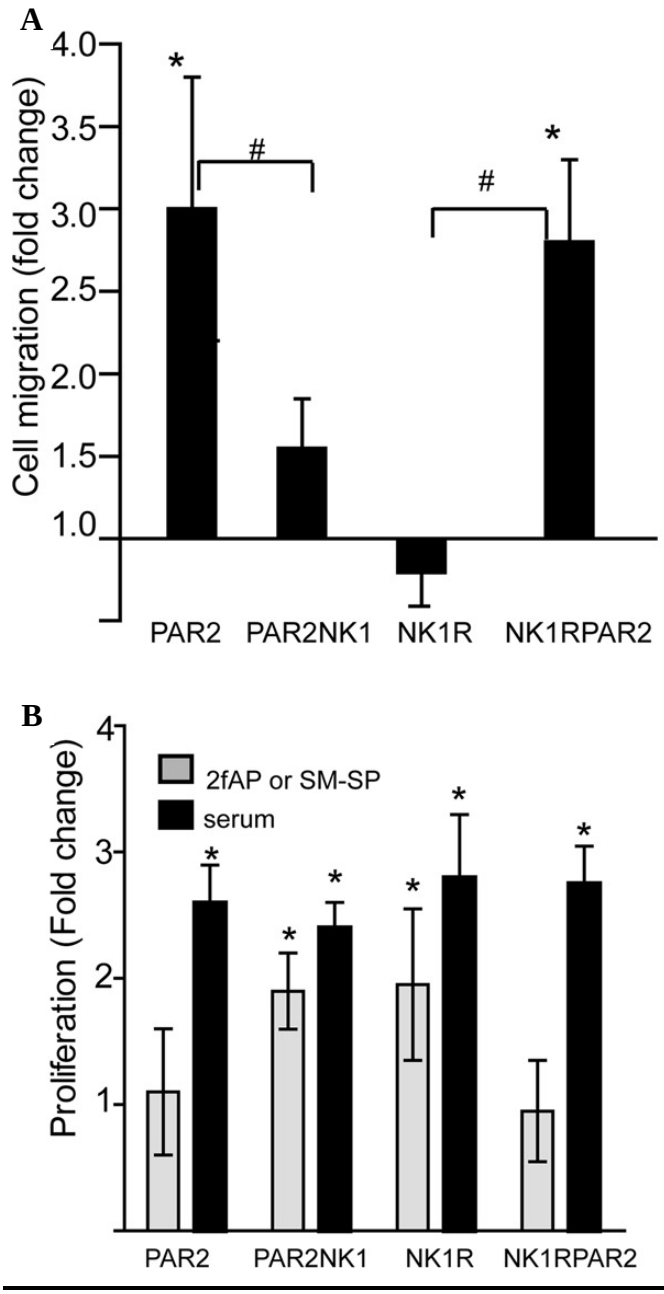

LAMP-1
Merge
PAR2
PAR2NK1R
NK1R
NK1RPAR2

Figure 2.8. Cytosolic distribution of activated ERK1/2 by the chimeric receptors are similar to the C-terminal parent receptor: GFP tagged wild type or chimeric receptor expressing CHO cells were stimulated with the respective agonists for 60 minutes and immunostained for pERK1/2. The cells were fixed, stained with DAPI and observed by confocal microscopy.

PAR2NK1R
NK1RPAR2
Merge
TO-PRO
pERK
PAR2
NK1R
0 min
60 min
0 min
60 min
0 min
60 min
0 min
60 min

Figure 2.9: β-arrestin dependent physiological outcomes of chimeric receptor activation are dependent on their C-terminal tails. HEK 293 cells were transiently transfected with wild type or chimeric receptors. (A) For cell migration assays, cells were seeded on 2uM transwell filters. Cell migration is expressed as an increase in the number of cells that migrate in response to 2fAP or Sar-Met-SubP stimulation compared to untreated cells. (B) For proliferation assays the transfected cells were serum starved and then stimulated with 2fAP, Sar-Met-SubP or serum for 24 hours. Fold increase in cell proliferation over untreated groups have been represented as mean ± SEM. ($p < 0.01$)


A
Cell migration (fold change)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
#
#
*
PAR2
PAR2NK1
NK1R
NK1RPAR2
B
Proliferation (Fold change)
4
3
2
1
2fAP or SM-SP
serum
*
*
*
*
*
*
PAR2
PAR2NK1
NK1R
NK1RPAR2

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CHAPTER 3:

Phosphorylated serine/threonine residues of Protease Activated Receptor 2(PAR2) C-terminal tail direct β-arrestin 1/2 recruitment and binding

ABSTRACT

Protease activated Receptor-2 (PAR2) is a G protein coupled receptor (GPCR) which signal via $G\alpha_q$ or $G\alpha_{12/13}$ pathway. Receptor activation by proteolytic cleavage of the N terminus leads to signaling by both G protein and β-arrestin dependent pathways. When signaling by the Gαq cascade, PAR2 can promote weak nuclear ERK1/2 activation. The classic paradigm for GPCR sequestration predicts that, ligand activated receptor is phosphorylated at the C terminal by second messenger kinases and G protein coupled receptor kinases (GRK), leading to β arrestin recruitment. G protein signaling by PAR2 is terminated by β-arrestin binding, which uncouples the receptor from G proteins and promotes internalization. β arrestin1/2 remain associated with PAR2, on endocytotic vesicles. Signaling by the β-arrestin-dependent pathway, leads to sustained activation of membrane associated ERK1/2 and cell migration. Furthermore, downstream of PAR-2, β-arrestins can scaffold and inhibit LIM-Kinase (LIMK), to activate the actin severing protein, cofilin. Stability of association with the receptor, appears to be important for β-arrestin-dependent signaling. The molecular determinants for this stable association between PAR2 and β-arrestins have not been elucidated. Clusters of phosphorylated serine and threonine residues in the C termini of a number of GPCRs direct stable β arrestin/receptor interactions. The PAR2 C-terminus possesses several such S/T clusters. BRET assays using pharmacological inhibitors of the Gq pathway showed that β-arrestin recruitment to PAR2 can take place independently of initial

Gq signaling. Using $PAR2^{WT}$, $PAR2^{S363/6A}$ (putative Protein kinase C mutant) and $PAR2^{0P}$ (phosphorylation null mutant) we observed that, β-arrestin 1/2 recruitment rates are attenuated by ~10 fold, in absence of PKC mediated C-tail phosphorylation. β-arrestin 1/2 recruitment to $PAR2^{0P}$ was completely abolished. While $PAR2^{WT}$ promotes robust cofilin activation and chemotaxis, $PAR2^{S363/6A}$ and $PAR2^{0P}$ fail to do so. Thus, although β-arrestin 1/2 recruitment to PAR2 C tail can take place in absence of initial Gq signaling, the C-tail needs to be phosphorylated, to ensure stable association of β-arrestin 1/2 with PAR2 C-tails.

INTRODUCTION

Protease activated receptor 2 (PAR 2) can be activated by a number of different serine proteases. These proteases range form trypsin, members of the blood coagulation cascade like, factors VIIa and Xa, mast cell tryptase(1,2) and also proteases released from tumor microenvironment like tissue factor plasminogen (3). Proteolytic cleavage generates a tethered ligand sequence (SLIGRL for mouse and SLIGKV for human)(4-6), which binds to the extracellular face of the receptor to activate it. Apart from naturally occurring proteases, PAR2 activation can also be triggered by synthetic peptide agonist of the tethered ligand sequence (7). Following activation, PAR2 can signal by multiple G proteins: $G_{\alpha q}$ or $G_{\alpha 12/13}$ to promote activation of a multitude of downstream effectors (8,9).

Signaling by the $G_{\alpha q}$ pathway leads to activation of the membrane bound enzyme Phospholipase C-β (PLC β). This breaks phosphatidylinositol-4,5-biphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} from intracellular sources and DAG activates Protein Kinase C (PKC) (10). This $G_{\alpha q}$ signaling pathway leads to weak nuclear ERK1/2 activation and has also been shown to activate PI3Kinase by involving Src (11,12). $G_{\alpha 12/13}$ signaling by PAR2 activates the monomeric small GTPase RhoA. PAR2 dependent RhoA activation can lead to cytoskeletal rearrangement to direct cell migration (8,9,13).

PAR2 signaling by the G protein pathway is terminated by cytosolic scaffolding molecules β-arrestin 1/2. β-arrestin 1/2 have been shown to desensitize, endocytose and traffic the internalized PAR2 to distinct sub-cellular compartments (14-16). The classic paradigm of GPCR downregulation requires that the C-terminal tails of agonist occupied receptor are phosphorylated by second messenger kinases (PKC or PKA) or G protein coupled receptor kinases (GRKs). While second messenger kinases mediate heterologous desensitization, GRKs carry out homologous desensitization (17,18). First identified GRK sites were a cluster of serine residues from positions 355 to 364 in the C-terminal of the β2-adrenergic receptor (19). GRK-5 plays a vital role in attenuating PAR1 signaling in endothelial cells (20). However, it is unknown whether PAR2 desensitization or phosphorylation is mediated by GRKs.

In addition to the classic G protein signaling pathways, PAR2 can trigger unique signaling pathways using the pleiotropic scaffolding molecules β-arrestin 1/2. It has been reported that PAR2 can elicit prolonged membrane ERK1/2 activation by β-arrestin 1/2 mediated scaffolding of MAPK module. Such sustained activation of ERK1/2 close to the membrane leads to rearrangement of actin cytoskeleton in cell cortex, which brings about cell migration (11,21). The PAR2 $G_{\alpha q}$ pathway leads to activation of the p110 catalytic subunit of PI3K. On the other hand β-arrestin 1/2 mediated scaffolding inhibits the PI3kinase activity both by direct binding as well as by inhibiting upstream molecule Cdc42 (12,22).

In addition, PAR2 uses the β-arrestin 1/2 mediated pathway to activate cofilin. Cofilin is an actin severing protein, which breaks down pre-existing filaments to generate, free barbed ends, thereby generating membrane protrusions. Cofilin is negatively regulated by the upstream enzyme LIMkinse (LIMK), by phosphorylation at the conserved third serine residue. On the other hand, HAD family phosphatase, chronophin (CIN) dephosphorylates cofilin to activate it (23). β-arrestin 1 can scaffold LIMK to inhibit it, thereby preventing cofilin inhibition. β-arrestin 1 has also been shown to bind CIN and cofilin together in a complex. This ensures spatial proximity of CIN towards cofilin to ensure cofilin activation by dephosphorylation. PAR2-β-arrestin1-cofilin-CIN complexes have been purified from pseudopodia of immune cells. This enables migration of cells to a chemotactic gradient (24,25).

This ability of β-arrestin 1/2 to act as molecular scaffolds enabling GPCRs to signal by non-traditional pathways, have been reported in a case of number of other receptors like gonadotropin releasing hormone receptor, parathyroid receptor, neurokinin-1-receptor (NK1R), type I angiotensin II receptor, vasopressin 2 receptor and the chemokine receptor CXCR4 and CCR7 (11,26-30). In case of the type IA angiotensin receptor, β2-adrenergic receptor and the parathyroid receptor the β-arrestin mediated ERK1/2 activation can operate independently of the G protein pathway (31-33). However, in case of PAR2 it is

still unknown whether β-arrestin recruitment or receptor internalization can be carried out independently of initial G protein signaling.

Because of the nature of PAR2 activation, i.e., by irreversible proteolytic cleavage of the N-terminus to generate a tethered ligand, which cannot diffuse away, mechanisms involved in receptor desensitization and downregulation are crucial determinants regulating kinetics and magnitude of cellular responses. As described before, phosphorylation of GPCR C-terminal tail is a key event for initiating the receptor desensitization process. PAR1 C-terminal tail have been reported to be phosphorylated in response to agonist treatment. Mutating a cluster of serine/threonine residues at the center of the C-terminal tail of PAR1, leads to prolonged signaling by the G protein pathway. However the duration of internalization and degradation of this PAR1 mutant is unaffected (34). This indicates that there exist specific roles for the different phosphorylation sites in PAR1 C-terminal tail. Such distinct roles of phosphorylated S/T residues have been reported in the follicle stimulating hormone (FSH) receptor. A cluster of 5 S/T site in the C-tail of FSH receptor has been reported to play a crucial role in desensitization. However, mutating this cluster does not affect β-arrestin dependent ERK activation (35).

Similar to PAR1 and FSH receptor, the C–tail of PAR2 also has multiple phosphorylation sites, including 18 S/T residues (36). By blocking PKC activity,

using pharmacological inhibitors it has been shown that this second messenger kinase play a vital role in PAR2 desensitization (37). A putative PKC mutant PAR2 (PAR2δS363/6A) was reported to carry out prolonged Ca^{2+} signaling, showed defective internalization and promoted nuclear translocation of activated ERK1/2 (11). A C-terminal phosphorylation nil mutant of PAR2 (PAR2 0P) has been reported to signal constitutively by G_q and its internalization was carried out by a non-canonical dynamin dependent but β-arrestin/clathrin independent pathway (36).

In the previous chapter we have reported how the C-tail of different GPCRs are vital determinants of receptor-β-arrestin stability as well as β-arrestin dependent regulation of signaling. Specific domains in the C-terminal of the human hydroxycarboxylic acid receptor 2 (HCA_2) have been shown to meadite distinct functions like cell surface expression, desensitization, internalization as well as maintaining the receptor in an active conformation (38).

No such investigation has been carried out to characterize the functional roles of specific residues in the PAR2 C-tail. The specific roles of phosphorylation of PAR2 C terminal by PKC and GRKs on β arrestin1/2 recruitment and their influence on β arrestin dependent signaling pathways such as membrane-ERK1/2 activation, cofilin activation, PI3kinase inhibition has not been looked into. Thus the goal of this project is to look into the specific roles of

PKC and GRK phosphorylation of PAR2 C-tail on β-arrestin dependent signaling pathways.

MATERIALS & METHODS

Materials

Unless mentioned all reagents were purchased form Sigma. The following primary antibodies were used for western blotting or on cell western assays: Mouse monoclonal antibodies to FLAG M2 (Sigma); rabbit anti phospho-cofilin (Cell Signaling); mouse anti total-cofilin (BD Transduction laboratories). IRDye®680 and IRDye®800 tagged secondary antibodies (Rockland) were used for western blotting and on cell western assays, to enable visualization using the LICOR odyssey imaging system. *2-furoyl*-LIGRLO-NH*2,* U73122 and BAPTA-AM were purchased from Tocris. FLAG tagged $PAR2^{WT}$, $PAR2^{S363/6A}$ and $PAR2^{0P}$ in the pBJ1 vector, Renilla Luciferase tagged β-arrestin 1 and 2 and GRK-2 constructs were obtained as gifts from Dr. JoAnn Trejo (UCSD), Dr. Michel Bouvier (University of Montreal) and Dr. Brad Anderson (Western University of Health Sciences) respectively. For BRET assays, FLAG-tagged $PAR2^{WT}$, $PAR2^{S2363/6A}$ and $PAR2^{0P}$ were subcloned from the pBJ1 vector into the p-eYFP-N1 vector using HindIII and BamHI. GRK-2 cDNA was subcloned into the Luciferase vector using Xho1 and BamH1.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cell lines were grown in 10% (v/v) fetal calf serum (FCS) supplemented Dulbecco's modified Eagle's media (DMEM) and maintained at 37°C and 5% CO_2. The cells were passaged using Cell stripper

solution (Cellgro). For BRET, cell migration and cofilin dephosphorylation assays, transient transfections were carried out in 80% confluent HEK 293 using FuGene6 (Roche) following the manufacturer's protocols.

Bioluminescence Resonance Energy Transfer (BRET)

β-arrestin1/2-RLuc or GRK2-Rluc and eYFP tagged receptor constructs were transiently co-expressed in HEK-293 cells. 24 hours after transfection, the cells were distributed in poly-lysine coated 96 well plates (white bottomed). 48 hours post transfection, the cells were treated with appropriate concentrations of 2fAP and 5uM coelenterazine. Readings were taken 15 minutes after agonist stimulation in case of dose response curves. When required, pharmacological inhibitors were added at appropriate concentrations and the cells were incubated at 37°C, prior to addition of 2fAP. Light emission was detected (460–500 nm for RLuc and 510–550 nm for YFP) using a TRISTAR LB941 multilabel plate reader from Berthold Technologies. BRET signal was calculated as the ratio of the light emitted by eYFP and the light emitted by luciferase. As negative control, cells transfected with the luciferase construct alone were used to determine the background. Half lives ($t_{1/2}$) of the kinetics reactions were determined from 5 separate experiments.

Western blotting

HEK 293 cells were transiently transfected with FLAG tagged $PAR2^{WT}$, $PAR2^{S363/6A}$ or $PAR2^{0P}$. 36 hours post transfection, the cells were serum starved overnight. When required the cells were treated with appropriate concentration of

inhibitors or vehicle for 10 mins. Following activation with 1uM 2fAP for 0-60 minutes, the cells were washed with 1X PBS and treated with cofilin lysis buffer (phosphate buffered saline pH 7.6, supplemented with 10mM NaF, 2mM Na_3VO_4, 1mM EGTA, 1% Triton-X100 and protease inhibitors). 30ug of the cleared protein lysates were loaded into the wells of 15% SDS-PAGE gel. Western blotting was carried out by transferring proteins onto PVDF-FL membrane (Millipore) and blocking in 1% fish gelatin in TBS. All primary antibodies were used at a concentration of 1:1000. The blots were scanned using LICOR Odyssey imaging system. The bands were quantified using the LICOR software. Western blot images were processed by Adobe Photoshop CS3 and placed in Adobe IllustratorCS3.

On cell Western assay

HEK293 were transiently transfected with FLAG tagged $PAR2^{WT}$, $PAR2^{S363/6A}$ and $PAR2^{0P}$. 24 hours post transfection, the cells were transferred to collagen coated 24 well plates and allowed to attach and grow overnight. The cells were fixed with 4% paraformaldehyde, blocked using Odyssey blocking buffer and incubated with mouse anti-FLAG monoclonal antibody (1:100) overnight at 4°C. Following incubation with IRDye®680 tagged secondary antibody (1:800), the plates were scanned using LICOR Odyssey imaging system, and the integrated intensities of the wells were quantified using the LICOR software.

Cell migration assay

HEK 293 cells transiently transfected with the FLAG tagged wild type or phosphorylation mutants of PAR2 were allowed to become confluent as continuous monolayer for 36 hours post transfection. The cells were then serum starved overnight and a wounding effect was generated by disrupting the monolayer with a pipette tip. The cells were subjected to 2fAP stimulation for 24hrs and the number of cells entering the wound from the adjoining area was quantified.

RESULTS

Published data in the field has shown that PAR2 can activate ERK1/2 both by using the classic G protein pathway and the novel β arrestin1/2 dependent signaling arm. While the G protein pathway requires Ca^{2+} dependent proline rich tyrosine kinase 2 (Pyk 2) and Src activation, the β arrestin1/2 pathway can function independently of Src. Thus, in case of PAR2 the two signaling cascades appear to function independently of each other (11,21,39). On the contrary, in case of NK1R both the G protein and the β arrestin1/2 dependent ERK1/2 activation requires activation of Ras and Src. Hence, it appeared that the two pathways are cooperative and did not antagonize each other as in case of PAR2 (40). Using Ca^{2+} chelator and pharmacological inhibitor of Src on wild type and β arrestin1/2 double knock out (DKO) mouse embryonic fibroblasts (MEF), these subtle mechanistic differences in ERK 1/2 activation were teased out. While PAR2 dependent ERK1/2 activation in wild type MEFs were reduced by only 30%, similar experiments in NK1R expressing MEFs showed a 95% drop in ERK1/2 activation (41). Thus it appeared that while PAR2 can signal independently of the initial G_q pathway using β arrestin1/2, NK1R requires integration of both the signaling arms.

Thus we went on to determine whether the initial G_q signaling is necessary for β arrestin1/2 recruitment to NK1R and PAR2. PKC one of the key effectors

generated in the G_q pathway establishes a feedback effect by phosphorylating the C-terminal tail of activated GPCRs. As described before, these phosphorylated S/T residues serve as a trigger for β arrestin1/2 recruitment. In order to determine whether initial G_q signaling is at all needed for β arrestin1/2 recruitment, we carried out BRET assays using pharmacological inhibitors of the effectors of G_q signaling pathway. We used BAPTA to chelate Ca^{2+}, GFX a broad-spectrum inhibitor of PKC and U73122, which inhibits PLCβ. Both β arrestin-1 and 2 were recruited to PAR2 when stimulated with 2fAP, in spite of pretreatment of the cells with the inhibitors. However, there was a nearly 50% drop in β arrestin1/2 recruitment to NK1R. These results indicated that unlike NK1R, PAR2 efficiently recruits both β arrestin1/2 even in absence of initial G_q signaling [Figure 3.1].

PKC is an important downstream effector generated by the G_q pathway, which has been shown to play a vital role in PAR2 desensitization (37). As inhibition of PKC did not abolish β arrestin1/2 recruitment, we went on to determine whether, the PAR2 C-terminal tail needs to be phosphorylated to facilitate β arrestin1/2 recruitment and binding. PAR2 C-tail has 18 potential phosphorylation sites. We designed a putative PKC mutant of PAR2 by mutating the serine and threonine residues at positions 363 and 366 to alanine (PAR2δ363/6A). A phosphorylation null mutant (PAR20P) was also designed by mutating all of the 18 S/T residues in the PAR2 C-tail.

Before these phosphorylation mutants were used in any cell based assays, we needed to confirm that these mutants were expressed at comparable levels at the cell surface like the wild type receptor. Cell surface ELISA using N-terminal FLAG tagged receptors confirmed that PAR2WT, PAR2δ363/6A and PAR20P are expressed at similar levels [Figure 3.2 (A)].

Next, BRET kinetics assays showed that β arrestin1/2 recruitment to PAR2δ363/6A was significantly attenuated by 2 fold and was completely abolished in PAR20P [Figure 3.2 (C-D)]. Furthermore, $BRET_{50}$ values were significantly enhanced by nearly 10 fold for the putative PKC mutant [Figure 3.2 (E-F)]. This indicated that the binding affinity of β arrestin1 and 2 for PAR2 C-terminal tail is significantly decreased in absence of PKC phosphorylation. $BRET_{max}$ values were also different for the PAR2 WT and PKC mutant. A higher $BRET_{max}$ value for WT receptor indicated that the luciferase tagged β arrestin1/2 interacts with the eYFP tagged PAR2 C tail in such a way that the donor and acceptor are oriented at closer proximity, compared to the PAR2δ363/6A receptor. Furthermore, we also observed that the putative PKC mutant of PAR2 binds to β arrestin1 with 2 fold greater affinity than β arrestin 2 [Figure 3.2 (E-F)].

Finally, we went on to determine whether these differences in β arrestin1/2 recruitment and binding affinities observed in case of the phosphorylation mutants, affected β arrestin1/2 dependent cofilin activation. Consistent with the

results of BRET assays, results of cofilin dephosphorylation assays showed that following 2fAP stimulation, PAR20P failed to promote cofilin activation by dephosphorylation, like the wild type receptor. However, to our surprise we observed that PAR2δ363/6A, which successfully recruited β arrestin1/2, although at significantly reduced rates compared to the wild type, was also unable to activate cofilin by dephoshorylation [Figure 3.3].

As β-arrestin-dependent cofilin dephosphorylation is required for PAR2 induced cell migration, we examined the ability of PAR2δ363/6A to promote cell migration using wound-scratch assays. Consistent with the inability of PAR2δ363/6A to promote β-arrestin-dependent cofilin activation, the PKC mutant was also defective in promoting cell migration, like the phosphorylation null mutant [Figure 3.4].

It appears that the PAR2-β-arrestin 1/2 scaffolding complex is less stable in case of PAR2δ363/6A, leading to poor cofilin dephosphorylation and cell migration. Thus, β-arrestins can be recruited to PAR2 in the absence of PKC phosphorylation. Phosphorylated S363 and T366 serve as anchors for stable PAR2-β-arrestin 1/2 association, which in turn regulates downstream cofilin signaling crucial for cell migration [Figure 3.6].

Since, β-arrestin-1/2 recruitment to the PAR20P mutant was completely abolished, we hypothesized that there, maybe unidentified kinases that may phosphorylate the PAR2 C-tail to trigger initial β-arrestin 1/2 recruitment. We decided to focus on the GRKs, as they have been shown to play vital roles in phosphorylating agonist occupied GPCRs to promote desensitization by β-arrestin 1/2 recruitment. We particularly focused on GRK2, as it possesses binding site for active G_q-GTP complex in the RGS homology domain. This disrupts G_q signaling by G_q coupled GPCRs. However this association has not been reported to enhance GTPase activity. Thus, GRK2 isoform is capable of providing another mechanism of desensitization in addition to its kinase activity whereby it establishes binding sites for β-arrestin 1/2 (42).

Results of BRET titration assays showed that there is specific binding between PAR2 and GRK2 as reflected by the hyperbolic rise in BRET ratio with increase in acceptor to donor ratio. We also observed a dose dependent incremental rise in GRK2 recruitment following 2fAP stimulation [Figure 3.5].

DISCUSSION

In this study we reported that β-arrestin 1/2 recruitment to 2fAP stimulated PAR2 can occur independently of initial G_q signaling, as opposed to closely related NK1R. Furthermore, we identified putative PKC phosphorylation sites at positions 363 and 366 in PAR2 terminal tail, which function as β-arrestin 1/2 binding sites. In absence of PKC β-arrestin 1/2 recruitment and binding is substantially reduced and β-arrestin 1/2 dependent cofilin activation and subsequent cell migration is completely abolished. In addition we also reported that β-arrestin 1/2 recruitment to PAR2 was completely abrogated in absence of phosphorylation. Finally, we went on demonstrate that GRK2 is recruited to PAR2 following agonist stimulation. It will be interesting to see whether GRK2 can phosphorylate PAR2 C-tail and also determine how GRK-2 mediated phosphorylation of PAR2 C-tail can affect β-arrestin 1/2 dependent regulation of PAR2 signaling.

The current investigation highlights the fact that differences in β-arrestin 1/2 mediated regulation of signaling by different GPCRs can be attributed to the specific phoshorylation sites in receptor C-tail. A previous study had reported that stability of phosphorylated GPCR- β-arrestin 1/2 complex is determined by the distribution and proximity of S/T clusters or potential phosphorylation sites in the C-terminal of the respective GPCRs. Using confocal live cell microscopy it

was shown in the study that neurotensin -1 receptor, NK1R, oxytocin receptor and angiotensin IA receptor which possess one or more clusters of S/T residues in the C-tail formed stable complex with β-arrestin 1/2 which persisted even on endosomes. On the other hand, β2-adrenergic receptor (β2AR) which lacks S/T clusters, shows a more transient association with β-arrestin 1/2. β-arrestin 1/2 do not colocalize with internalized β2AR on endosomal vesicles (43).

More recently, biased agonists of certain GPCRs have been shown to favor a specific pathway over the other. Stimulation of CCR7 by endogenous ligands CCL19 and CCL21 promotes distinct functional consequences. Whereas CCL19 alone can promote β-arrestin 2 mediated receptor internalization and classical receptor desensitization, CCL21 fails to do so. These differences were due to the distinct GRK isoforms that were activated. While CCL19 activated both GRK3 and GRK6, CCL21 exclusively activated GRK6. Thus, the differences in receptor phosphorylation profiles that were generated elicited differences in β-arrestin 1/2 dependent functions (44).

This has been very well highlighted in case of vasopressin receptor (V2R). Using siRNAs to knockdown GRK2, 3, 5 and 6 it was demonstrated that the different GRK isoforms have distinct roles to play in regulating signaling by V2R. GRK2 and 3 phosphorylate agonist occupied V2R and recruit β-arrestin 1/2. GRK 5 and 6 were largely involved in β-arrestin 1/2 dependent ERK1/2 activation (45).

Similar, GRK isoform specific receptor phosphorylation establishes a “bar-code” for β-arrestin 1/2 functions even in case of β2AR. The phosphorylation “bar-code” ensures that β-arrestin 1/2 bind to the receptor C-tail with distinct conformations, depending on which of the S/T sites have been phosphorylated. This in turn affects, which of the downstream binding partners can be a part of GPCR-β-arrestin 1/2 signaling scaffold (46).

Activation of PAR2 by certain agonists promotes inflammation, pain sensation, changes in epithelial permeability and cell migration (47). PAR2 signaling is known to play key roles in a number of pathological conditions like cancer, asthma and arthritis (48). Accumulating evidence from different sources suggest that PAR2 antagonists and biased agonist have enormous potential as therapeutic tools. Thus a clear understanding of the mechanisms of PAR2 membrane trafficking, phosphorylation, desensitization and internalization is essential. Each of these processes can potentially initiate or terminate specific signaling cascades. This will enable us to design novel drugs and thereby manipulate signaling via PAR2.

Studies on the interaction between receptor and downstream signaling molecules like β arrestins is important, as erroneous formation of such complexes may lead to pathological consequences. Impaired interaction between β arrestins and mutant V2R has been reported to cause nephrogenic diabetes insipidus (49). Published data indicates that prolonged interaction between rhodopsin and visual

arrestin maybe an underlying cause for retinal degeneration (50,51). Thus, insights into the molecular determinants of PAR2 and β arrestin complexes will help us to identify mechanisms and drugs to manipulate PAR2 responsiveness.

FIGURES & LEGENDS

Figure 3.1: Inhibition of Gαq signaling does not inhibit β arrestin 1 or 2 recruitment to agonist stimulated PAR2. HEK 293 cells transiently transfected with PAR2-eYFP (A) or NK1R-eYFP (B) and β arrestin 1/2-Rluc were pretreated with BAPTA (30μM, 10min), GFX (10μM, 10min) and U73122 (10μM, 10min) and then stimulated with 2fAP (A) (1μM, 20mins) or Sar-Met-SP (B) (100nM 20mins). Net BRET values with and without agonist treatment were determined and graphed as the change in net BRET in the presence and absence of agonist (induced Net BRET). (*, $p<0.05$. **, $p<0.002$)

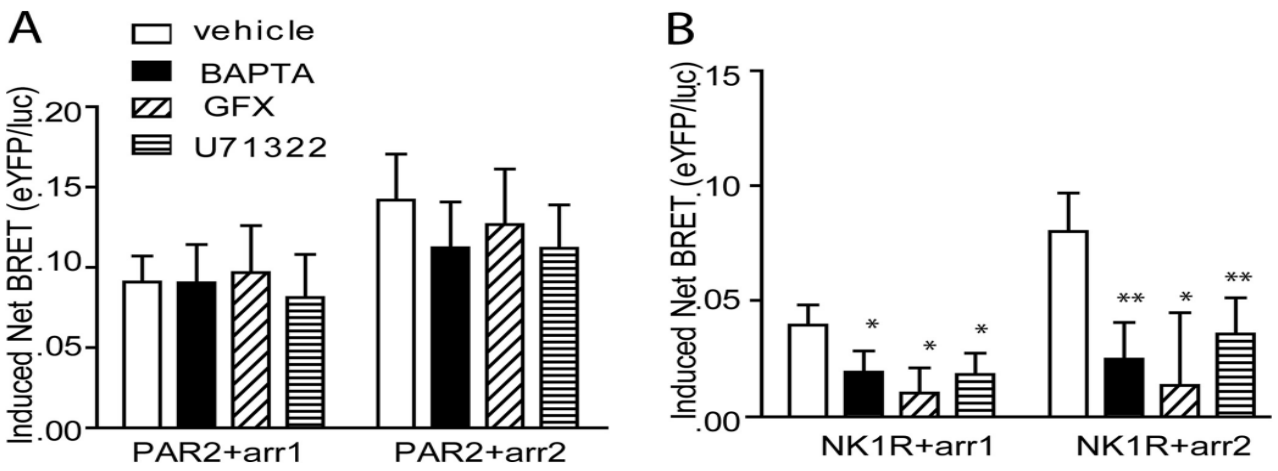

A
vehicle
BAPTA
GFX
U71322
Induced Net BRET (eYFP/luc)
.20
.15
.10
.05
.00
PAR2+arr1
PAR2+arr2
B
Induced Net BRET (eYFP/luc)
.15
.10
.05
.00
*
*
*
**
*
**
NK1R+arr1
NK1R+arr2

Figure 3.2. β arrestin 1/2 recruitment to wild PAR2 and phosphorylation mutants $PAR2^{S363/6A}$ and $PAR2^{0P}$: (A) Estimation of cell surface expression of N-terminal FLAG tagged $PAR2^{WT}$, $PAR2^{S363/6A}$ and $PAR2^{0P}$ in transiently transfected HEK 293 cell lines, by on cell western using anti FLAG monoclonal antibody. (B) BRET assays were carried out to monitor Rluc-β arrestin-1/2 recruitment using C-terminal eYFP tagged wild and phosphorylation mutants of PAR2. Net BRET ratios were quantified in response to incremental doses of 2fAP. Inset represents percentage of maximal BRET response to increasing concentrations of 2fAP. (C) Net BRET ratios were monitored in response to 1uM 2fAP over a period of 20 min. (D) Half lives ($t_{1/2}$) of BRET kinetics assays shown as mean ± SEM. (E) Net BRET ratios were monitored in response to titration of YFP tagged receptors to a constant amount of Rluc- β arrestin-1/2. Table 1: $BRET_{50}$ and $BRET_{max}$ values were computed from the titration curve in (E).

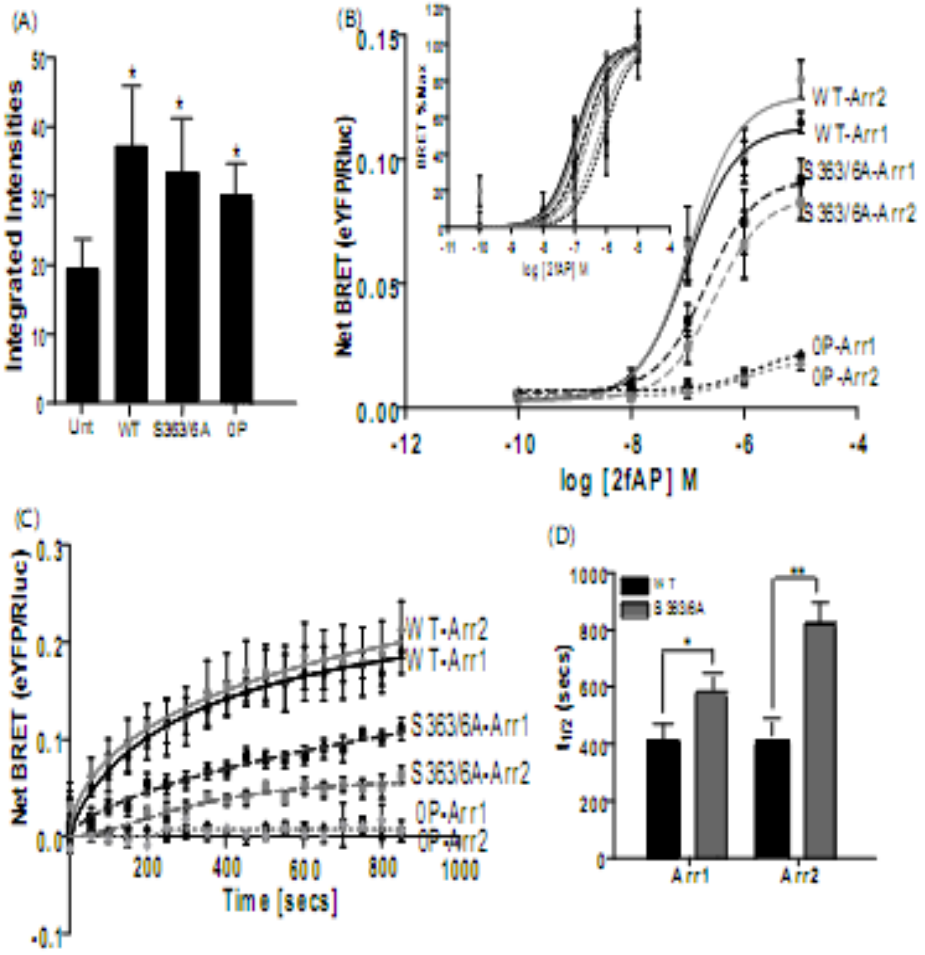


TABLE 1.

	WT/Arr1	WT/Arr2	STa/Arr1	STa/Arr2	0P/Arr1	0P/Arr2
Bmax	0.2136	0.2125	0.08623	0.09590	0.01518	0.009083
BRET 50	0.2248	0.1416	2.127	4.910	2.485	3.387

Figure 3.3: PAR2$^{S363/3A}$ and PAR2^{0P} fails to promote cofilin dephosphorylation. HEK 293 cells were transiently transfected with FLAG tagged PAR2^{WT}, PAR2^{0P} and PAR2$^{S363/6A}$. (A) Following activation with 2fAP, the cells lysates were subjected to Western blotting for phosphorylated and total cofilin. (B) Time course of phospho-cofilin generation expressed as fold increase over total-cofilin. The results are mean ± SE of 3 independent experiments.

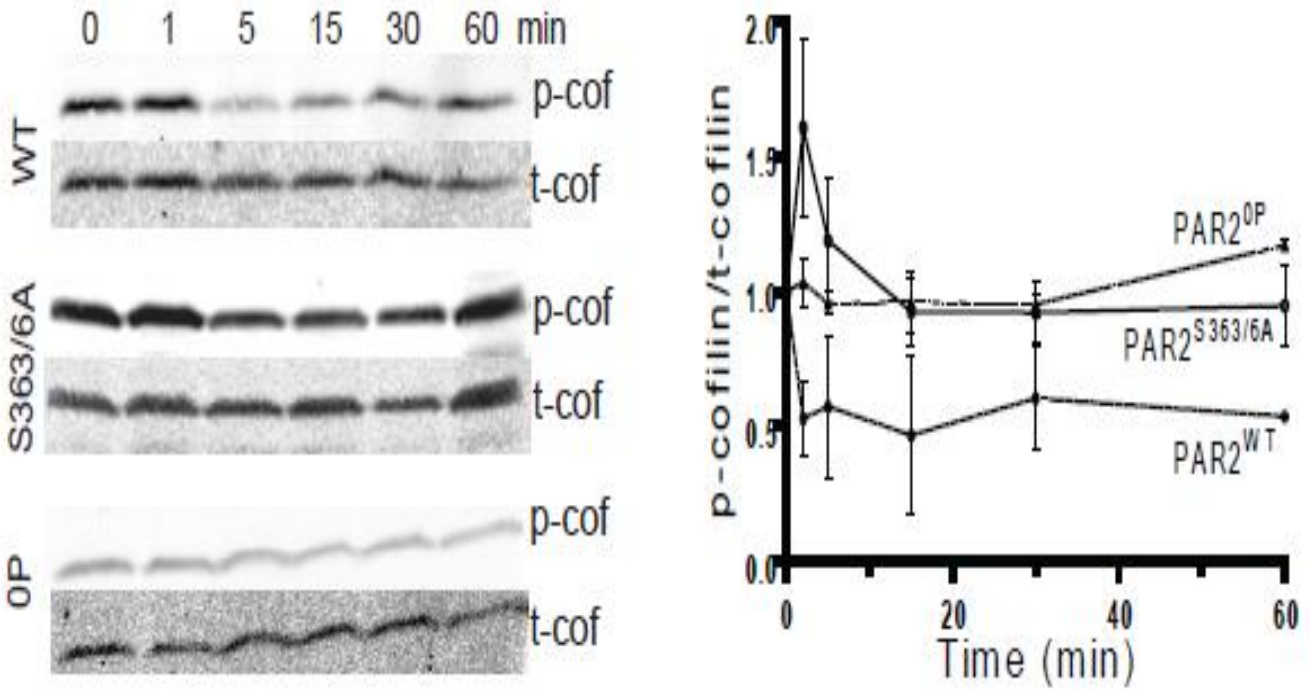

0 1 5 15 30 60 min
WT
p-cof
t-cof
S363/6A
p-cof
t-cof
0P
p-cof
t-cof
p-cofilin/t-cofilin
2.0
1.5
1.0
0.5
0.0
0
20
40
60
Time (min)
PAR2^0P
PAR2^S363/6A
PAR2^WT

Figure 3.4. PAR2 phosphorylation mutants are unable to promote cell migration following 2fAP stimulation: Wild type and phosphorylation mutant PAR2 transfected cells were grown to form a continuous monolayer. The confluent cells were disrupted to create a wounding effect and treated with 2fAP. 24 hours post activation, the number of cells which migrated to the wounded area were counted for 2fAP treated and untreated cells for each of the receptor subtypes. The fold increase in cell migration for 2fAP treated cells over untreated cells was computed and represented as mean ± SE.

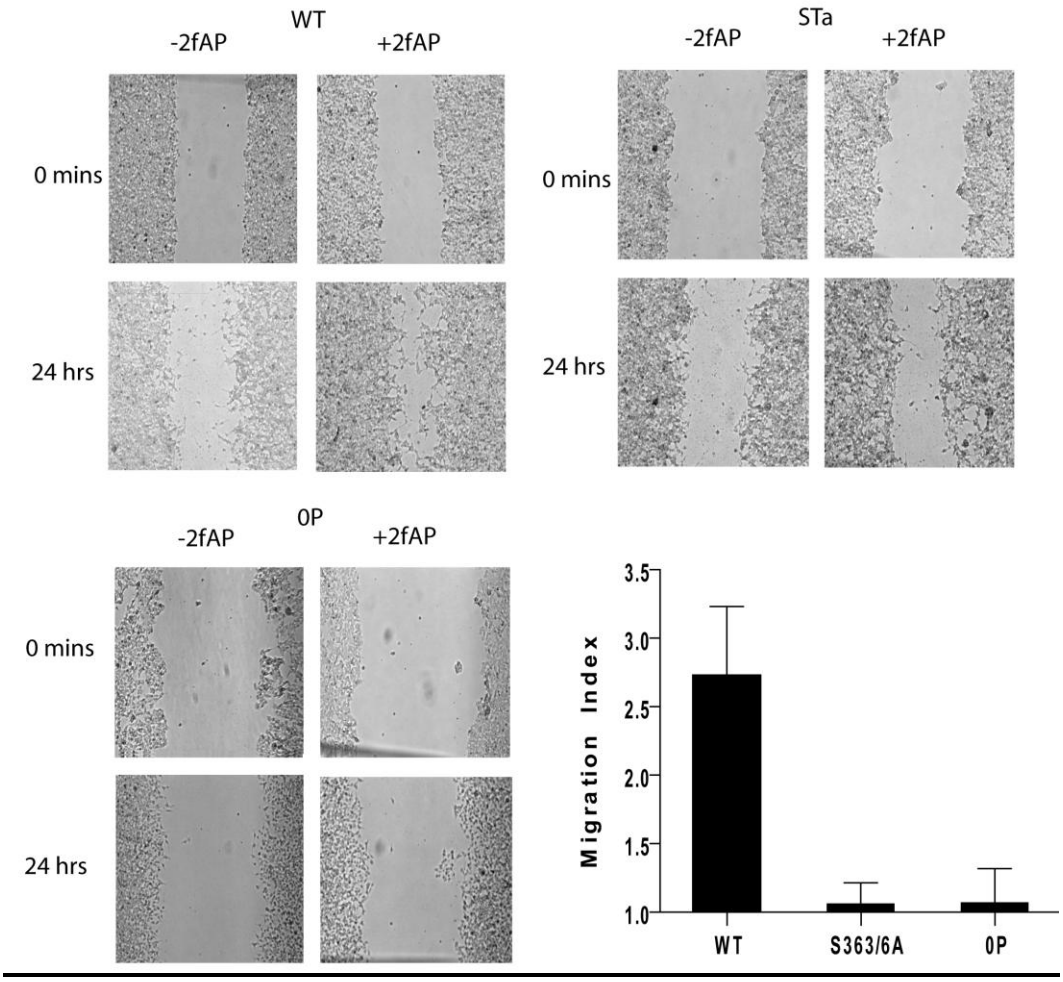

WT
-2fAP
+2fAP
0 mins
24 hrs
STa
-2fAP
+2fAP
0 mins
24 hrs
0P
-2fAP
+2fAP
0 mins
24 hrs
Migration Index
3.5
3.0
2.5
2.0
1.5
1.0
WT
S363/6A
0P

Figure 3.5: GRK-2 is recruited to PAR2 following 2fAP stimulation: HEK-293 cells transiently transfected with PAR2-eYFP and Rluc-β arrestin-1/2 were subjected 2fAP stimulation. (A) Net BRET values were plotted as a function of increasing acceptor to donor ratios. (B) Net BRET values were plotted as a function of increasing concentrations of 2fAP.

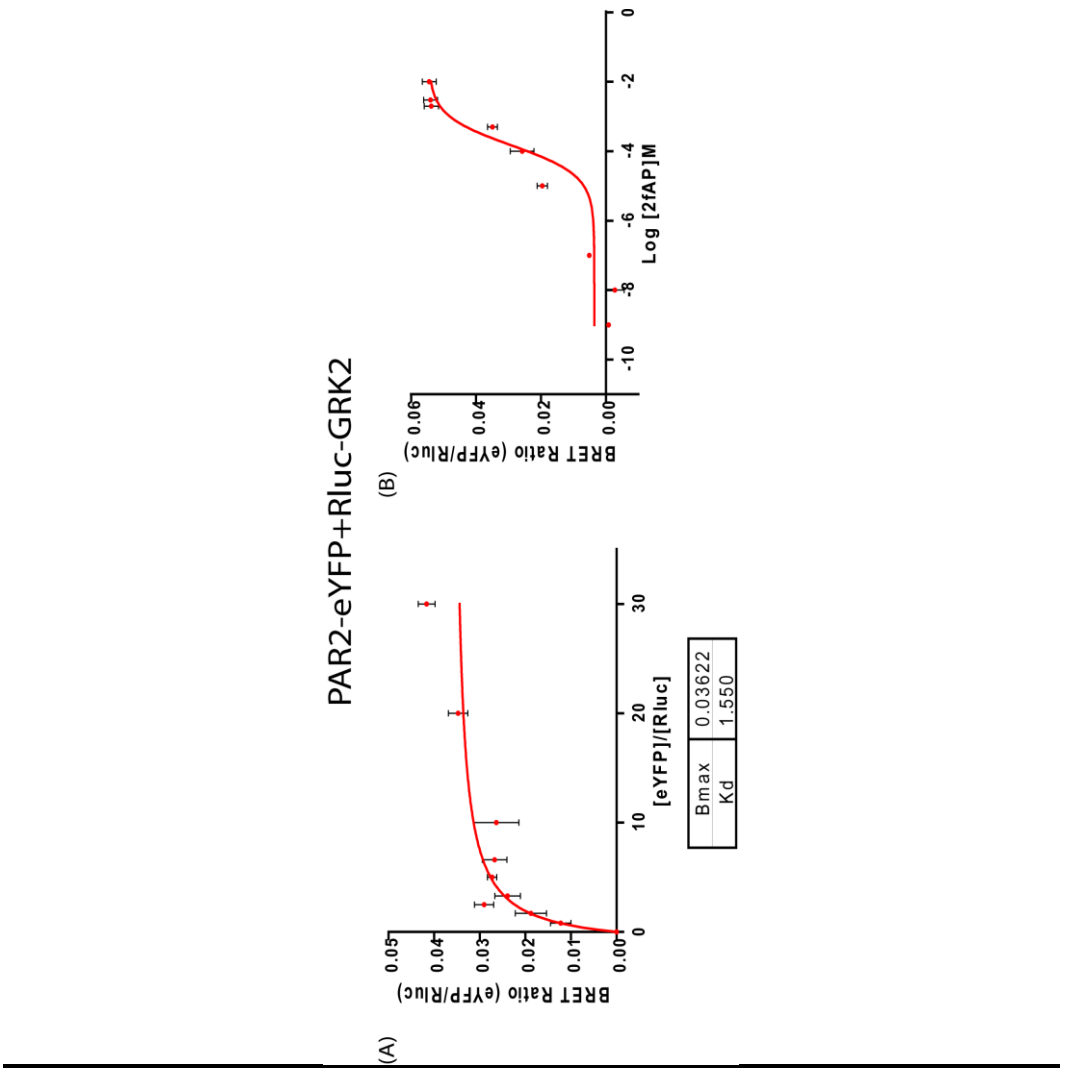
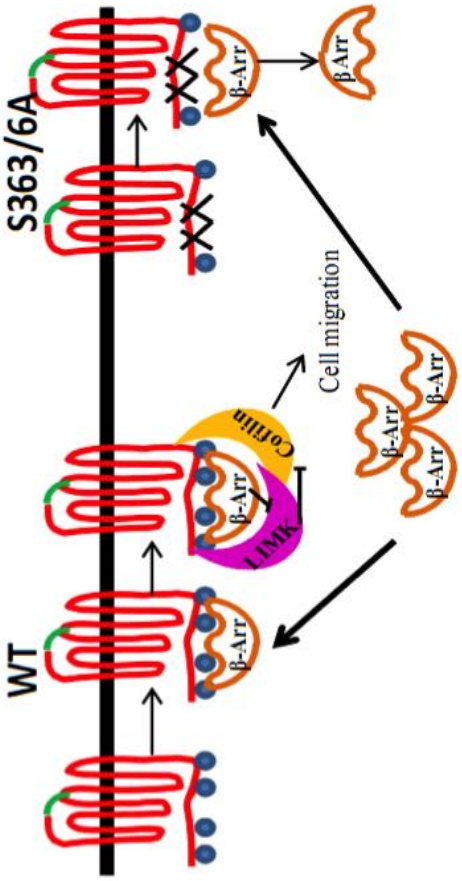

PAR2-eYFP+Rluc-GRK2
(A)
BRET Ratio (eYFP/Rluc)
0.00
0.01
0.02
0.03
0.04
0.05
0
10
20
30
[eYFP]/[Rluc]
Bmax 0.03622
Kd 1.550
(B)
BRET Ratio (eYFP/Rluc)
0.00
0.02
0.04
0.06
0
-2
-4
-6
-8
-10
Log [2fAP]M

Figure 3.6. Role of phosphorylated serine and threonine residues of PAR2 C-terminal tail in β arrestin recruitment and binding: Phosphorylation of specific S/T residues in the PAR2 C-tail by unknown kinases is necessary for β-arrestin-1/2 recruitment. β-arrestin 1/2 recruitment to PAR2 C-tail persists in the absence of Protein Kinase C (PKC) phosphorylation, at significantly attenuated rates. PKC mediated phosphorylation at positions 363 and 366 establishes docking sites for β-arrestin binding. In absence of PKC phosphorylation, PAR2-β-arrestin association is not stable, which abolishes β-arrestin dependent cofilin activation and subsequent cell migration.


S363/6A
WT
β-Arr
β-Arr
β-Arr
β-Arr
β-Arr
β-Arr
β-Arr
Cofilin
LIMK
Cell migration

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CHAPTER 4:

Allergic proteases from *Alternaria alternata* and *Blatella germanica* promote β-arrestin dependent cofilin signaling by PAR2

4.1 ABSTRACT

Protease activated receptor 2 (PAR2) is extensively expressed on the airway epithelia and the cells of the immune system, thus making it an important candidate for allergic asthma characterized by airway hypersensitiveness and pro-inflammatory symptoms. PAR2 activation by serine proteases leads to signaling by 2 opposing pathways: The classic $G_{\alpha q}$ pathway, which leads to protective effects and the unique β-arrestin-1/2 pathway, which mediates the pro-inflammatory effects. Allergic proteases from the fungus *Alternaria alternata* and the household pest *Blatella germanica* leads to PAR2 signaling by the canonical $G_{\alpha q}$ pathway. The current project was undertaken to determine whether these pathogenic proteases can lead to PAR2-β-arrestin signaling to promote cell migration of immune cells, which is characteristic of the pro-inflammatory effect. Results of BRET assays using cultured cell lines coupled with cofilin dephosphorylation and transwell migration assays using primary leukocytes from wild type and β-arrestin-2 knock out mice indicate that the allergic proteases utilize the PAR2-β-arrestin-2 signaling axis to promote cell migration by cofilin activation. Thus generation of biased antagonists, which preferentially inhibit the β-arrestin pathway is important for treatment of asthma.

4.2 INTRODUCTION

Protease activated receptor 2 (PAR2) is extensively distributed in the intestine, vascular and non vascular smooth muscles, airway epithelial cells, leucocytes as well as in primary sensory afferent neurons and astrocytes in the central nervous system (1,2). In each of these tissue types PAR2 can be activated by a number of different tissue types.

Mast cells have been implicated in airway fibro-proliferative diseases by activating PAR2 using mast cell tryptase. Furthermore, it has been characterized that tryptase activated PAR2 leads to enhanced expression of cyclooxygenase 2 (COX2). COX2 in turn leads to activation of prostaglandin E2 (PGE2). Consequently PGE2 promotes cell proliferation using peroxisome proliferator activated receptor γ (PPRγ) (3,4).

Trypsin generated by tumor micro-environment leads to PAR2 dependent colon cancer cell proliferation through activation of mitogen activated protein kinases (MAPK) and transactivation of Epidermal Growth Factor receptor (EGFR) (5,6). PAR2 activation on the basolateral surface of intestinal epithelial cells by trypsin has been involved in enhanced ion transport (7).

PAR2 cleavage by tissue factor/factor VIIA complex leads to migration of vascular smooth muscle cell migration (8). Activation of PAR2 expressed on rat

knee joint dorsal root ganglia (DRG) cells leads to increased firing rates, leukocyte rolling and adhesion (9). Inflammatory proteases like, neutrophil elastase, cathepsin G and proteinase 3 have been reported to disarm PAR2 and prevent activation of PAR2 signaling by trypsin. However, only elastase can lead to MAPK activation by PAR2 in cultured mouse embryonic firbroblasts, which is independent of β-arrestin 1/2 (10). However, neutrophil proteinase-3 has been reported to activate PAR2 in human non-epithelial cells to promote generation of inflammatory cytokines (11).

Apart from endogenous proteases, PAR2 can also be activated by protease secreted by pathogenic and allergic microbes. An arginine protease secreted by *Porphyromans gingivalis* can activate PAR2 expressed on oral epithelial cells to induce interleukin-6 (IL-6) production (12). House dust mite protease can activate PAR2 in human primary nasal epithelial cells to promote activation of apical chloride (Cl^-) channels. Through this study, PAR2 has been implicated in mucociliary clearance and hypersecretion of fluids in response to allergens (13). In-vivo studies in mice using 2fAP, has shown enhanced leucocyte migration, with a significantly higher percentage of eoisinophils in bronchoalveolar lavage fluid (BALF) as well as hyper-responsiveness (AHR). However, these pathological responses, which are indicative of allergic asthma, were significantly reduced by greater than 70% in PAR2 knock-out mice (2).

Very recently serine proteases from the household mold *Alternaria alternata* have been reported to activate PAR2 signaling by the classic G_q pathway, in bronchial epithelial cell line and PAR2 transfected HeLa cells. Activation of Ca^{2+} signaling by PAR2 using *A. alternata* extracts (AE) is similar to that observed in case of synthetic ligands like SLIGRL-NH_2 or 2fAP. Furthermore, in vivo experiments in mouse revealed that, AE generated protease dependent lung inflammation, by recruitment of immune cells in BALF. Exaggeration of these conditions may lead to allergic asthma (14).

Proteases from the German cockroach, *B. germanica* (CE) also generated allergic lung inflammation in mouse. Intranasal challenge using CE generated airway AHR and inflammation. Antibody mediated blockage of PAR2 in the sensitization phase, completely abolished AHR and allergic responses. PAR2 activation by CE persists even in TLR4 knock out mouse indicating there is no cross-talk between TLR4 and PAR2 signaling pathways through NFκB. Absence of allergic responses in TLR4 knock out mouse also indicated that the inflammatory responses generated by CE was not mediated by LPS, but exclusively involved protease action (15,16).

Although, the previous two studies by Boitano *et al.*, and Arizmandi *et al.*, has implicated that the $G_{\alpha q}$ signaling arm is activated by the allergic proteases, it remains unknown whether the β-arrestin 1/2 signaling arm plays a role in any of

the pathogenic responses. β-arrestin 2 has been shown to play a crucial role in allergic asthma. Chemokine driven immune cell migration to lung tissues and airway obstruction is a hallmark of asthma patients (17). β-arrestin 2 has been shown to play a role, in T-lymphocyte migration in airways. β-arrestin 2 knock out mice do not show lymphocyte accumulation in BALF, inflammatory responses and other pathophysiology associated with asthma (18).

Consistent with this finding, a recent study from the laboratory has shown that the $G_{\alpha q}$ signaling arm and the β-arrestin 2 pathways mediate seemingly opposing responses downstream of PAR2, in a mouse ova-induced allergic asthma model. While the canonical $G_{\alpha q}$ signaling arm mediates protective effects like bronchiolar smooth muscle relaxation, β-arrestin 2 signaling leads to inflammatory responses (19).

Although distinct physiological responses have been attributed to the divergent signaling arms of PAR2, the mechanism by which β-arrestin 2 elicits immune cell migration, in response to pathogenic proteases remains yet to be elucidated. The current study is directed towards dissecting the molecular mechanisms that govern β-arrestin 2 dependent immune cell migration in response to PAR2 activation by allergic proteases from *Alternaria alternata* and *Blatella germanica*.

4.3 MATERIALS & METHODS

Materials

Unless mentioned all reagents were purchased form Sigma. *Alternaria alternata* and *Blatella germanica* extracts were purchased from Greer laboratories. The following primary antibodies were used for western blotting: rabbit anti phospho-cofilin (Cell Signaling); mouse anti total-cofilin (BD Transduction laboratories. AF546 tagged secondary antibody to mouse and rabbit were obtained from Invitrogen. IRDye®680 and IRDye®800 tagged secondary antibodies (Rockland) were used for western blotting and on cell western assays, to enable visualization using the LICOR odyssey imaging system. *2-furoyl*-LIGRLO-NH*2* was purchased from Tocris. Trypsin was purchased from Worthington Biochemicals.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cell line was grown in 10% (v/v) fetal calf serum (FCS) supplemented Dulbecco's modified Eagle's media (DMEM) and maintained at 37°C and 5% CO_2. The cells were passaged using Cell stripper solution (Cellgro). For BRET, transient transfections were carried out in 80% confluent HEK 293 using FuGene6 (Roche) following the manufacturer's protocols. Spleen lymphocytes obtained from wild type or β-arrestin 2 knock out mice were grown in RPMI-1640 and maintained 37°C and 5% CO_2.

Bioluminescence Resonance Energy Transfer (BRET)

β-arrestin1/2-RLuc and eYFP tagged PAR2 constructs were transiently co-expressed in HEK-293 cells. 24 hours after transfection, the cells were distributed in poly-lysine coated 96 well plates (white bottomed). 48 hours post transfection, the cells were treated with appropriate concentrations of 2fAP, trypsin, AE, CE and 5uM coelenterazine. Readings were taken 15 minutes after agonist stimulation in case of dose response curves. Light emission was detected (460–500 nm for RLuc and 510–550 nm for YFP) using a TRISTAR LB941 multi-label plate reader from Berthold Technologies. BRET signal was calculated as the ratio of the light emitted by eYFP and the light emitted by luciferase. As negative control, cells transfected with the luciferase construct alone were used to determine the background. Half lives ($t_{1/2}$) of the kinetics reactions were determined from 5 separate experiments.

Western blotting

4×10^5 spleen lymphocytes from wild type or β-arrestin 2 knock out mice were seeded in 35 mm dishes and allowed to attach for 4 hours. Following activation with trypsin, AE or CE for 0-60 minutes, the cells were washed with 1X PBS and treated with cofilin lysis buffer (phosphate buffered saline pH 7.6, supplemented with 10mM NaF, 2mM Na_3VO_4, 1mM EGTA, 1% Triton-X100 and protease inhibitors). 30ug of the cleared protein lysates were loaded into the wells of 15% SDS-PAGE gel. Western blotting was carried out by transferring proteins onto PVDF-FL membrane (Millipore) and blocking in 1% fish gelatin in TBS. All

primary antibodies were used at a concentration of 1:1000. The blots were scanned using LICOR Odyssey imaging system. The bands were quantified using the LICOR software. Western blot images were processed by Adobe Photoshop CS3 and placed in Adobe IllustratorCS3.

Cell Migration assay

$2x10^4$ spleen lymphocytes were transferred to collagen coated 5μm (6.5mm diameter) trans-well supports and allowed to attach for 2 hours at 37°C. Following addition of AE, CE or 2fAP, to the lower chambers of the permeable supports, the cells were incubated at 37°C for 4 hours. The total number of cells migrated to the bottom of the filter were stained with crystal violet and quantified by under 20X objective of Nikon phase contrast microscope.

4.4 RESULTS

PAR2 activation by proteolytic cleavage with trypsin and mast cell tryptase has been reported to promote β-arrestin dependent signaling (20,21). Although AE and CE mediated PAR2-$G_{\alpha q}$ signaling has been demonstrated, role of β-arrestins in downstream signaling by AE and CE remains unexplored. Hence we decided to see whether activation of PAR2 by AE and CE also leads to β-arrestin-1/2 recruitment.

Results of BRET assays show that both β-arrestin 1 and 2 are recruited to PAR2 C-tail in a dose dependent fashion. The EC_{50} values provide the efficacy of the different proteases to promote β-arrestin-1/2 recruitment to PAR2. Results indicated that the ability of the synthetic peptide mimic of the PAR2 tethered ligand 2fAP to activate PAR2-β-arrestin 1/2 is similar to that observed for AE, i.e., AE like 2fAP appears to recruit both β-arrestins with equal affinity. On the contrary, CE preferentially recruits β-arrestin-1 over -β-arrestin-2. On computing the EC_{50} values, we observed that CE is a more potent agonist of PAR2 dependent β-arrestin recruitment as indicated by its 10 fold lower EC_{50} value [Figure 4.1 (C-F)].

Furthermore, results of BRET titration assays show that activation of PAR2 by the allergic proteases, leads to specific interaction between β-arrestin

1/2 with the C-tail, as indicated by the hyperbolic rise in the BRET ratio with concomitant increase in acceptor-donor ratio [Figure 4.1 (A-B)].

Previous work in from the laboratory has shown that PAR2 can scaffold β-arrestin, coflin and chronophin in primary leukocytes (22). Once we confirmed that AE and CE could bring about successful β-arrestin recruitment, we went on to determine whether these proteases are also capable of β-arrestin dependent cofilin activation. To test this hypothesis, we established primary cultures of mouse splenic lymphocytes from wild type (WT) and β-arrestin 2 knock out (β2KO) mice, as β-arrestin 2 has been previously shown to be an important mediator of allergic asthma in an ova-induced murine model of asthma (19). Results of cofilin dephosphorylation assays using these primary lymphocytes indicated that, both AE and CE promoted rapid cofilin activation within one minute of treatment in wild type cells. However, no robust cofilin dephosphorylation was observed in β-arrestin-2 knock out cells [Figure 4.2].

Finally, transwell migration assays were carried out using splenic lymphocytes from WT and β2KO to determine whether chemotaxis in response to the allergic proteases is a β-arrestin 2 dependent function. We observed a 3.5 and 6 fold increase in cell in migration of WT cells over β2KO cells, in response to AE and CE treatment respectively [Figure 4.3].

4.5 DISCUSSION

PAR2 is extensively expressed in the airway epithelia and glands and has been implicated airway hyper-responsiveness and allergic asthma (23). Very recently, the divergent signaling properties of the $G_{\alpha q}$ and β-arrestin dependent signaling arms that operate downstream of PAR2 has been shown to be involved in regulating varying physiological responses in a murine model of allergic asthma. While the β-arrestin-2 dependent signaling arm promotes inflammatory responses by promoting migration of leucocytes to the airway, the classic $G_{\alpha q}$ pathway is involved in smooth muscle relaxation (19).

While the previous work employed the synthetic peptide agonist 2fAP, the current study, aims at dissecting the signaling pathways triggered by the more clinically relevant allergic proteases from *Alternaria alternata* and *Blatella germanica*. It will be interesting to see whether these pathogenic proteases utilize the similar signaling cascades as the synthetic peptide mimic or the conventional endogenous serine proteases that are known to activate PAR2.

Although the role of these proteases in activating the $G_{\alpha q}$ pathway has been explored, there is currently no data on the ability of these proteases to activate the non-cannonnical PAR2-β-arrestin signaling cascade. BRET assays in cultured HEK293 cell line indicated that AE and CE could bring about robust β-

arrestin-1/2 recruitment, like the trypsin positive control. Finally, using spleen leucocytes from WT and β2KO mice, we went on to show that AE and CE can promote β-arrestin-2 dependent cofilin activation and cell migration.

In the experiments that have been carried out so far, we have used mouse primary leucocytes. Eosinophils infiltrating into the airway is a characteristic of allergic asthma. Neutrophils on the other hand are indicative of severe acute asthma. Once present in the airway these granulocytes are known to release inflammatory mediators like myeloperoxisidase, metalloproteases, lactoferrin and elastase. All of these pro-inflammatory agents go on to reduce pulmonary functions (24-27). It is important to characterize the subtype of white blood cells that are being activated by AE and CE. Better insights into the cell types involved in mediating immune responses to allergic proteases, will help to design suitable drugs with fewer side effects.

PAR2 can be activated by a number of different bacterial proteases like those from *Porphyromonas gingivalis, Citrobacter rodentium, P. gingivalis* and *Serratia marcescens*, which leads to disease pathology extending from peridontis to infectious colitis. In most of these cases PAR2 activation leads to expression of the anti-microbial peptide β-defensin 2, pro-inflammatory cytokines like IL-8 and IL-6 as well as activation of the transcription factor NF-kB, which is widely involved in innate immunity (28-31). The ability of PAR2 to activity NF-kB, has

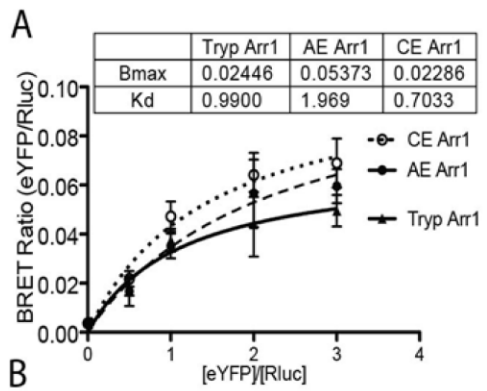
been reported to be a result of co-operative its interaction with Toll like receptor 4 (TLR4) in cultured HEK 293 cell lines (16).

Activation of PAR2 in the WT but not the β2KO mice leads to activation of IL-6 and TNF-α, which are characteristic of the Th2 responses of allergic asthma (19). Activation of these two pro-inflammatory cytokines have been observed downstream Myd88 activation in the TLR4 signaling pathway (32). Determining whether PAR2 co-operates with TLR4 to generate Th2 responses in allergic asthma is an important future pursuit.

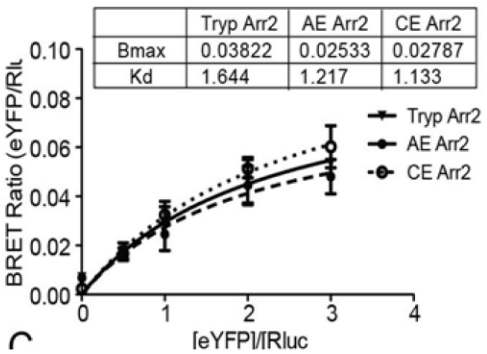
4.6 FIGURES & LEGENDS

Figure 4.1: PAR2 activation by AE and CE triggers β-arrestin recruitment.

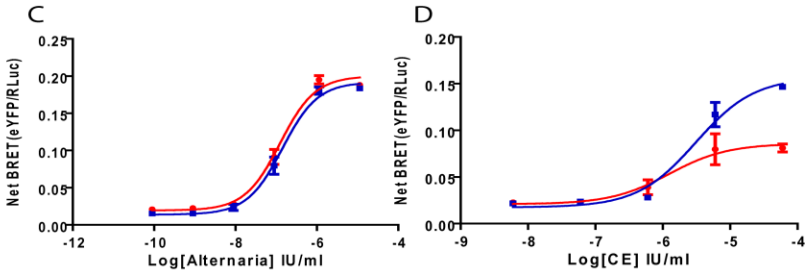
HEK 293 cells transiently transfected with PAR2-eYFP and β-arrestin-Rluc were stimulated using AE, CE and trypsin. (A-B) Net BRET ratios were estimated as a function of increasing acceptor/donor ratios, in response to AE, CE or trypsin treatment. $BRET_{50}$ and $BRET_{max}$ were calculated from the BRET titration curves. (C-E) Net BRET ratios were monitored in response to incremental concentrations of AE, CE and trypsin. Table -1 shows the EC_{50} values that were computed from the dose response curves in (C-E).



	Tryp Arr1	AE Arr1	CE Arr1
Bmax	0.02446	0.05373	0.02286
Kd	0.9900	1.969	0.7033



	Tryp Arr2	AE Arr2	CE Arr2
Bmax	0.03822	0.02533	0.02787
Kd	1.644	1.217	1.133



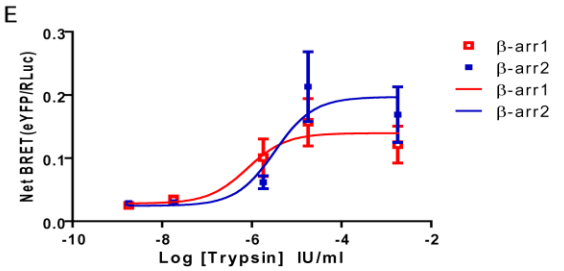


Table 1

	β-Arr1	β-Arr2
AE	1.253e-007	1.431e-007
CE	1.245e-006	3.105e-006
Trypsin	7.951e-007	3.255e-006

Figure 4.2: AE and CE promote cofilin activation using a β-arrestin-2 dependent pathway. (A-B) Primary leucocytes isolated from wild type and β-arrestin-2 knock out mouse were treated with AE, CE or trypsin for the indicated time points and the cells were subjected to western blotting for phosphorylated cofilin. (C) Amount of cofilin dephosphorylation generated by the respective protease was quantified and expressed as a fraction of baseline for wild type and β-arrestin-2 knock out cell types.

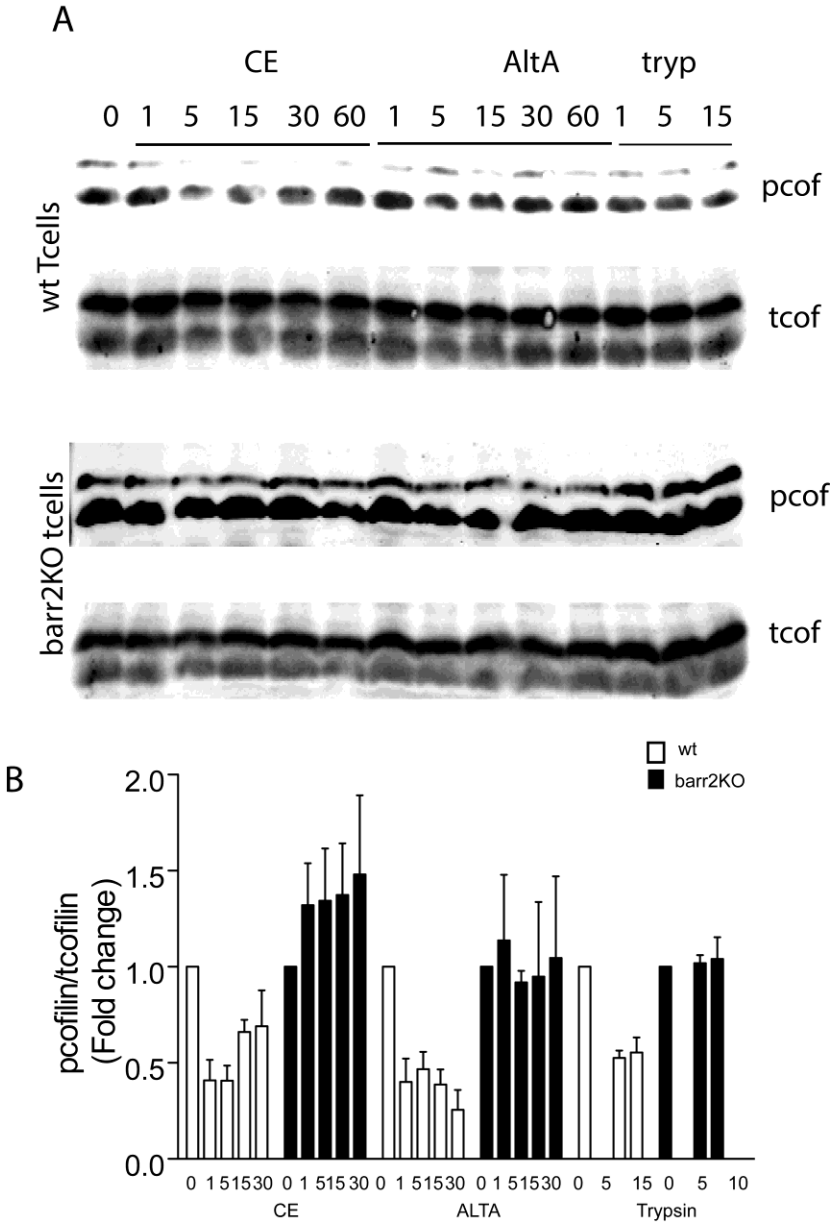
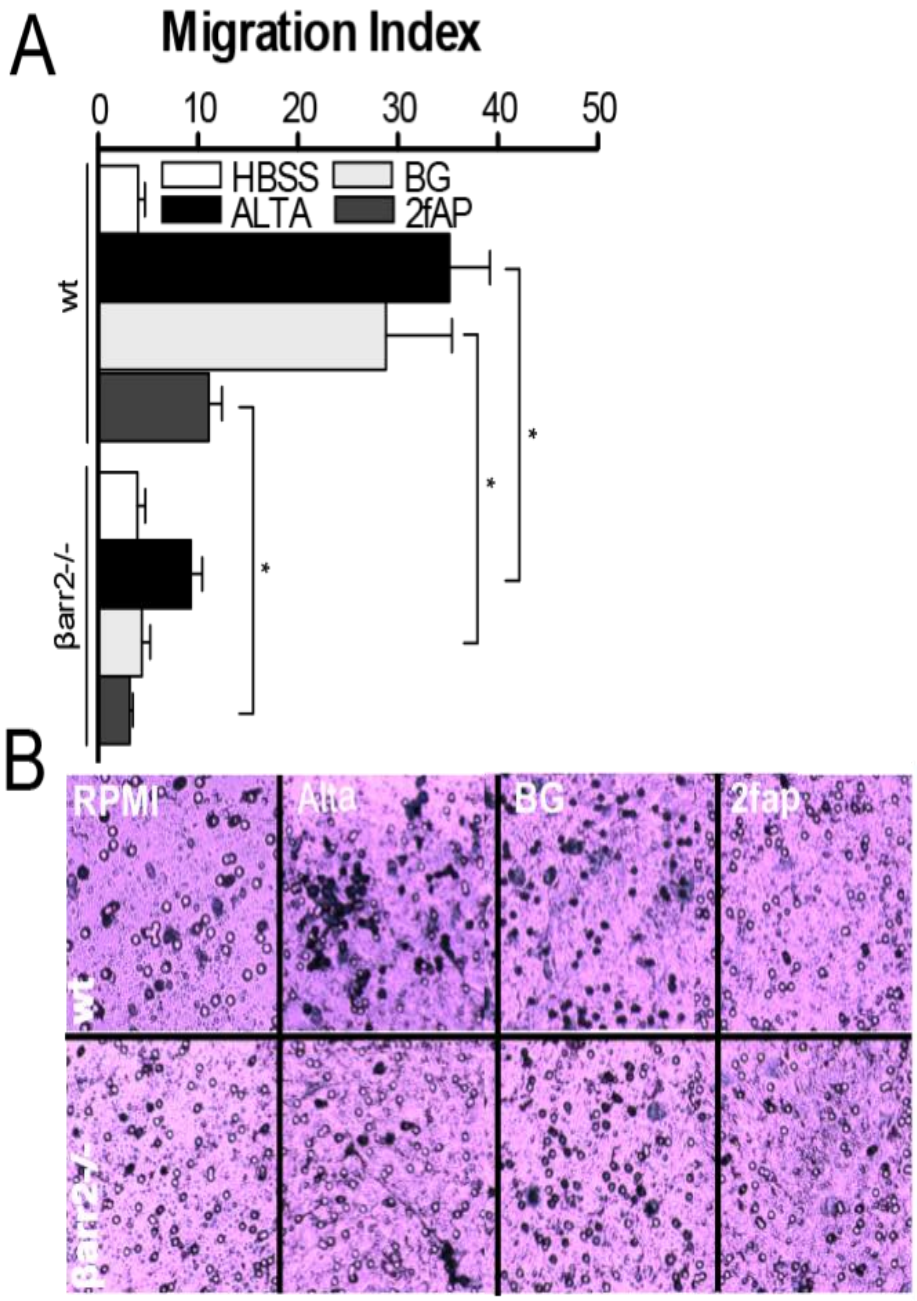

A
CE
AltA
tryp
0 1 5 15 30 60 1 5 15 30 60 1 5 15
wt Tcells
pcof
tcof
barr2KO tcells
pcof
tcof
B
wt
barr2KO
pcofilin/tcofilin
(Fold change)
2.0
1.5
1.0
0.5
0.0
0 1 5 15 30 0 1 5 15 30 0 1 5 15 30 0 1 5 15 30 0 5 15 0 5 10
CE
ALTA
Trypsin

Figure 4.3. AE and CE treatment to mouse spleen leukocytes leads to β-arrestin-2 dependent cell migration: Mouse spleen lymphocytes were seeded onto 5um transwell filters, which were left submerged in a lower chamber of RPMI containing AE, CE, 2fAP or HBSS for 4 hours and the number of cells migrating to the bottom of the filter in response to the agonist were calculated. (A) Mean ± SEM of the number of cells, which were attached to the underside of the transwell filters in response to agonist treatment. (B) Representative image of crystal violet stained leucocytes attached to the underside of transwell filters.



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Chapter 5:

Conclusions & Perspectives

PAR2 and NK1R are $G_{\alpha q}$ coupled 7 transmembrane receptors. While PAR2 is activated by irreversible cleavage of extracellular N terminus, NK1R activation requires reversible binding of the soluble ligand Substance P. However, the desensitization of the two receptors follows a similar pattern. This is involves binding of the cytosolic scaffolding proteins β-arrestins 1/2 to the C –tail of the receptor. This generates steric hindrance to uncouple the receptor- $G_{\alpha q}$ association. β-arrestins 1/2 also binds components of the cellular endocytotic machinery to package and internalize the receptors in clathrin coated pits. Following internalization the two receptors differ in their unique β-arrestin 1/2 dependent pathways. Both the receptors lead to ERK1/2 activation by these pathways. However, the difference in the components of the β-arrestin scaffolding complex, leads to membrane retention of ERK1/2 whereas in case of NK1R, ERK1/2 is translocated to the nucleus. We hypothesized that the, difference in β-arrestin1/2 dependent pathways in the case of the two GPCRs depend on nature of interaction of β-arrestin1/2 with the C-tail of the receptors.

In this investigation we have shown that activated NK1R demonstrates prolonged Ca^{2+} signaling compared to PAR2. Chimeric receptors, generated by switching the C-termini of the receptors shows the opposite effect, i.e., 2fAP activated PAR2NK1R has a longer duration of Ca^{2+} signaling than SP stimulated NK1RPAR2. After comparing receptor internalization patterns, we determined that agonist activated PAR2 internalizes more rapidly than NK1R. PAR2NK1R

on the other hand follows the slower internalization rates of NK1R. On the contrary NK1RPAR2 is rapidly removed from the membrane like its C-terminal parent. These properties of receptor desensitization and internalization are mediated by β-arrestin1/2 and these distinct patterns can be attributed to nature of β-arrestin association with the C-tail of the respective receptor. Both the wild type and the chimeric receptors are capable of ERK1/2 activation. However, the subcellular localization of activated p-ERK1/2 the chimeric receptors follows the C-tail parent. Cofilin dephosphorylation assays revealed that NK1R fails to activate cofilin unlike PAR2. Thus, PAR2 and NK1R show distinct trends with regards to β-arrestin mediated desensitization as well as signaling.

Results of BRET kinetics assays show that PAR2 recruits both β-arrestin 1 and 2 at a much more rapid rate compared to NK1R. This explains the difference in the desensitization and internalization rates in case of PAR2 and NK1R. We have also shown that NK1R preferentially recruits β-arrestin -2 at a significantly faster rate than β-arrestin -1. It has been previously reported that β-arrestin-1 binds to internalized PAR2 and targets it for lysosomal degradation. Although it has not been determined whether β-arrestin-1 plays the same role in case of other GPCRs, it maybe possible that NK1R is not directed to lysosomes, as β-arrestin-1 association takes place at a later time point. Also the stability of the NK1R/ β-arrestin-1 complex is weak, which might lead to dissociation of β-arrestin-1.

We also looked into two β-arrestin1/2 dependent signaling pathways, i.e., the ERK1/2 and cofilin pathways. While all four of the receptors were capable of ERK1/2 activation, the subcellular location of activated ERK1/2 differed. Activation of PAR2 and NK1R-PAR2 leads to prolonged membrane retention of p-ERK1/2. On the contrary, NK1R and PAR2/NK1R translocates activated ERK1/2 to the nucleus. Furthermore, NK1R fails to promote cofilin dephosphorylation unlike PAR2. This can be explained on basis of the $BRET_{50}$ values calculated from the BRET titration curve. The $BRET_{50}$ values for PAR2-β-arrestin 1/2 are significantly lower than NK1R-β-arrestin 1/2. This means that β-arrestin-1/2 has a higher affinity for the PAR2 C-tail than NK1R. Thus, the scaffolding complex formed in case of PAR2 is more stable than in case of NK1R. This can explain why ERK1/2 activated by PAR2-β-arrestin-1/2 scaffold is held at the membrane. As the affinity between NK1R and β-arrestin-1/2 is lower the scaffolding complex possibly gets dislodged from the receptor tail and enters the nucleus.

In addition, our current study also goes on provide a possible reason why PAR2 recruits β-arrestin-1/2 at faster rate than NK1R. Results of BRET assays showed that 2fAP activated PAR2 can recruit β-arrestin-1/2, independently of the classic $G_{\alpha q}$ pathway. NK1R on the other hand, failed to recruit β-arrestin-1/2 when the components of the $G_{\alpha q}$ pathway were blocked using pharmacological inhibitors. Following activation, NK1R generates PKC through the $G_{\alpha q}$ pathway,

which phosphorylates the receptor to initiate β-arrestin-1/2 association. GRK 2/3 is known to actively phosphorylate NK1R, which recruits β-arrestin-1/2 to the membrane. However, there are no reports of GRK phsophorylation in PAR2 desensitization. This difference may account for slower rates of β-arrestin-1/2 recruitment in case of NK1R, as it takes longer for GRK 2/3 to establish β-arrestin-1/2 binding sites by phosphorylation.

Keeping this in mind, we tried to determine whether it is necessary for PAR2 to be phosphorylated at it C-tail to ensure β-arrestin-1/2 recruitment and binding. There have been several reports showing PKC activation and inactivation leading to rapid desensitization and prolonged Ca^{2+} signaling by PAR2 respectively. Using the putative PKC mutant PAR2S363/6A, it has been shown that β-arrestin-1/2 fails to colocalize with the former at the membrane, making it internalization defective. Interestingly, results of BRET kinetics assays show that PAR2S363/6A recruits β-arrestin-1/2. However, it does so at significantly slower rates compared to the wild type receptor. $BRET_{50}$ values for PAR2S363/6A and β-arrestin-1/2 obtained from the BRET titration assays is also significantly higher. Thus the association between the C-tail of PAR2S363/6A and β-arrestin-1/2 is weaker and possibly a transient phenomenon. β-arrestin-1/2 molecules are possibly, recruited to the C-tail of activated PAR2S363/6A, however as the association is very weak, the arrestin molecules are easily dislodged. Hence it takes longer time for the saturation phase of the reaction to set in. This explains

the extreme right shift of the PAR2S363/6A-β-arrestin-1/2 BRET kinetics curve. Because of the unstable association of β-arrestin-1/2 with PAR2S363/6A, colocalization between these two proteins was not observed in a previous report. The $BRET_{max}$ values obtained for the PAR2S363/6A- β-arrestin-1/2 association are also significantly different from PAR2 wild type. This indicates that the conformation with which β-arrestin-1/2 molecules interact with PAR2S363/6A is different from the wild type receptor. In the BRET assays with the pharmacological inhibitors, we observed comparable $BRET_{max}$ values for the inhibitor treated as well as the untreated cells. This is because C-tail of PAR2 wild type used to transfect the cells in these experiments, fold in a particular conformation. However, the C-tail of PAR2S363/6A possibly has a different conformation, because of the point mutations. To bypass this effect, β-arrestin-1/2 molecules which are being recruited to this receptor, has to adopt a different conformation. Hence this leads to a different $BRET_{max}$ value compared to PAR2 wild type, as observed in the BRET titration assay.

PAR2S363/6A also fails to promote robust cofilin dephosphorylation like the wild type receptor. This is possibly because, cofilin activation is a β-arrestin-1/2 dependent phenomenon. As β-arrestin-1/2 does not associate with PAR2S363/6A stably it is unable to scaffold LIMK to inhibit it. In our experiments we also used $PAR2^{0P}$, which lacks all the potential phosphorylation

sites in its C-tail. BRET assays show, that it is not able to recruit β-arrestin-1/2. As a consequence, it is not able to activate cofilin.

Based on our results so far, we conclude that unlike NK1R β-arrestin-1/2 recruitment to PAR2 is independent of the initial G-protein signaling. PKC generated by this process, phosphorylates the C-tail of PAR2 at positions 363 and 366 to generate potential binding sites for β-arrestin-1/2. However, it appears that PAR2 C-tail needs to be phosphorylated by kinases other than PKC to trigger β-arrestin-1/2 following receptor activation. We decided to focus on the G protein coupled Receptor Kinase (GRK) family of serine/threonine kinases as they mediate homologous desensitization of a number of GPCRs. The GRK family comprises of 7 different isoforms, i.e., GRK 1-7. Using BRET we have shown that GRK-2 is specifically binds to PAR2 in a dose dependent fashion. However, it is still unknown whether GRK2 recruitment to PAR2 is essential for establishing phospho sites for β-arrestin binding or whether it serves any other purpose.

In the future studies are being undertaken to determine the whether GRK-2 can mediate PAR2 phosphorylation. Identification of phosphorylated serine/threonine residues in PAR2 using mass spectroscopy is also an important future pursuit. We are also interested in determining whether, knock down of GRK-2 leads to disruption of β-arrestin-1/2 recruitment to PAR2 or any of the β-

arrestin-1/2 dependent signaling pathway. Finally, using β-arrestin-1/2 double knock out mouse embryonic fibroblasts we would like to determine whether GRK-2 alone is sufficient to terminate Gq signaling. Furthermore we are also interested in determining, whether GRK-2 can mediate PAR2 internalization, as this serine/threonine kinase is known to possess a clathrin binding motif its Regulator of G protein Signaling homology (RH) domain.

Finally, using pathologically relevant allergic proteases to activate PAR2, we have shown that the β-arrestin dependent cofilin pathway leads to leucocyte migration. Infiltration of immune cells to lungs leads to inflammation as well as airway remodeling, which are the hallmarks of allergic asthma. Further studies are needed to determine whether there are crosstalks between the PAR2 signaling pathway and other signaling cascades of the immune system to bring about this effect. Clear understanding of these pathways, are necessary to design anti-inflammatory drugs targeted towards PAR2.