Study of ERK1/2 MAP kinases activation

by the bradykinin type 2 receptor:

Characterization of beta-arrestin scaffolding function in the temporal regulation of ERK1/2 activation induced by the B2R

By

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Abstract

G protein-coupled receptors (GPCRs) comprise the largest family of transmembrane receptors. The beta-arrestins, adaptor proteins involved in GPCR desensitization, may also act as scaffolds for signaling pathways such as the mitogen-activated protein kinase (MAPK) cascade. The MAPK family, which includes the extracellular-signal regulated kinases (ERK) 1 and 2, promotes cellular differentiation and proliferation. Herein, the activation of ERK1/2 upon stimulation of the GPCR bradykinin type 2 receptor (B2R) with bradykinin was examined. Various B2R mutants with modified C-termini were employed to examine the temporal kinetics of ERK1/2. One of these receptor mutants displayed a loss of beta-arrestin binding as well as greatly enhanced ERK1/2 activation, compared to the wild-type receptor, when a cluster of serine/threonine residues important for B2R internalization was mutated. The other receptor mutants exhibited a correlation between their affinity for beta-arrestin and the intensity of ERK1/2 activation. Data from a mouse embryonic fibroblast cell line null for beta-arrestin suggested that beta-arrestin is involved in late-phase ERK1/2 activation by the B2R. These data point to the involvement of beta-arrestin in the activation of the ERK1/2 MAPKs through the B2R.

Résumé

Les récepteurs couplés aux protéines G (RCPG) composent la plus grande famille de récepteurs trans-membranaires. Les beta-arrestines sont des protéines adaptatrices impliquées dans la désensibilisation des RCPG. Ils peuvent également servir de d'échafaudage pour la signalisation comme dans le cas des voies mitogéniques (mitogen-activated protein kinase (MAPK)). La famille des MAPK, qui inclut les extracellular-signal regulated kinases (ERK) 1 et 2, promouvoit les signaux mitogéniques ainsi que la différenciation et la prolifération cellulaires. Jusqu'à présent, peu de chose sont connues sur le rôle des beta-arrestines comme molécules d'échafaudages dans l'activation des voies MAPK induites par les récepteurs de la bradykinine de type 2 (B2R). Nous avons utilisé plusieurs mutants du récepteur B2 et des beta-arrestines afin d'examiner les cinétiques temporelles d'activation des ERK1/2 induites par la bradykinine. Un de ces récepteurs mutants a démontré une perte de liaison avec les beta-arrestines, et a été plus efficace dans l'activation de l'ERK1/2, par rapport au récepteur de type sauvage. De plus nous avons observé une corrélation entre l`avidité de différents autres récepteur B2 et beta-arrestines mutants, et l'intensité avec laquelle ceux-ci activent ERK1/2. Des résultats obtenus dans une lignée de fibroblastes embryoniques de souris déficientes pour la beta-arrestin suggèrent que la beta-arrestin, a travers B2R, serait impliquée dans l'activation a long terme de ERK1/2. Ces résultats suggèrent donc la participation des beta-arrestines dans l'activation des MAPK par le B2R.

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Abbreviations

1. AC – adenylyl cyclase
2. ADP – adenosine diphosphate
3. Ang II – angiotensin II
4. AP-2 – adaptor protein 2
5. ARF6 – ADP-ribosylation factor 6
6. ARNO – ARF nucleotide binding site opener
7. AT1R – angiotensin II type 1 receptor
8. AT2R – angiotensin II type 2 receptor
9. ATP – adenosine triphosphate
10. β2-AR – β2-adrenergic receptor
11. β-arrestin$_2$T381 – β-arrestin$_2$ truncated at amino acid 381
12. B1R – bradykinin type 1 receptor
13. B2R – bradykinin type 2 receptor
14. B2R5A – B2 receptor with residues at positions Thr-342, Thr-345, Ser-346 and Ser-348 mutated to Ala
15. B2RV2CT – chimeric mutant consisting of the human B2 receptor (amino acids 1-324) fused to the C-terminus of the human V2 receptor (amino acids 343-371)
16. B2RV2CT6A – B2RV2CT chimera with residues at positions Ser-362, Ser-363, Ser-364, Thr-369, Ser-370 and Ser-371 mutated to Ala
17. BK – bradykinin
18. cAMP – cyclic adenosine monophosphate
19. CFP – cyan fluorescent protein
20. C-terminus – carboxyl-terminus
21. DAG – diacylglycerol
22. E/DRY – Glu/Asp-Arg-Tyr
23. ECL – extracellular loop
24. EGFR – epidermal growth factor receptor
25. ERK – extracellular signal-regulated kinase
26. FRET – fluorescence resonance energy transfer
27. G protein – guanine nucleotide-binding protein
28. GAP – GTPase-activating protein
29. GDP – guanosine diphosphate
30. GEF – guanine nucleotide exchange factor
31. GIRK – G protein-gated inwardly rectifying potassium channel
32. GPCR – G protein-coupled receptor
33. GRK – G protein-coupled receptor kinase
34. GTP – guanosine triphosphate
35. HEK293 – human embryonic kidney 293
36. ICL – intracellular loop
37. IP3 – inositol 1,4,5-trisphosphate
38. JNK/SAPK – c-Jun N-terminal kinase/stress-activated protein kinase
39. kDa – kilodalton
40. KKS – kallikrein-kinin system

41. LARG – leukemia-associated RhoGEF
42. MAPK – mitogen-activated protein kinase
43. MAPKK – mitogen-activated protein kinase kinase
44. MAPKKK – mitogen-activated protein kinase kinase kinase
45. MEF – mouse embryonic fibroblast
46. MEK – MAP/ERK kinase
47. NEAK – Asn-Glu-Ala-Lys
48. NPXXY – Asn-Pro-x-x-Tyr; x = any amino acid
49. NSF – *N*-ethylmaleimide-sensitive fusion
50. N-terminus – amino-terminus
51. PIP2 – phosphatidylinositol 4,5-bisphosphate
52. PKA – protein kinase A
53. PKC – protein kinase C
54. PLC-β – phospholipase C-β
55. RGS – regulator of G protein signaling
56. RhoGEF – guanine nucleotide exchange factor for Rho
57. SH – Src homology
58. siRNA – small interfering RNA
59. TM – transmembrane
60. V2R – vasopressin type 2 receptor
61. YFP – yellow fluorescent protein

1. Introduction

Signal transduction is the process by which changes in extracellular information are translated into intracellular language that the cell can understand. It conveys information on numerous physiological functions such as cell proliferation, differentiation and death, release of neurotransmitters and hormones, and gene expression. G protein-coupled receptors (GPCRs) are a family of cell-surface proteins that play crucial roles in such signaling events (Nambi and Aiyar 2003).

GPCRs comprise the largest class of membrane-bound receptors. The members of this superfamily of heptahelical receptors are involved in a wide range of physiological processes, such as mediating the action of neurotransmitters, chemo-attractants, cytokines and hormones, as well as sensory stimuli (light, taste, pain and odor) (Pierce, Premont et al. 2002). Considering the large variety of important physiological processes that they are involved in, as well as the pathological states that may occur as a result of their aberrant function, it is of no surprise that GPCRs are attractive targets for therapeutic drugs. More than 50% of therapeutic drugs on the market directly or indirectly target GPCRs. GPCRs are coded by one of the largest gene superfamilies, representing more than 1% of the human genome (~800-1,000 genes) (Nambi and Aiyar 2003).

These ubiquitous receptors were named "G protein-coupled" because of their common feature of associating with heterotrimeric G proteins which bind guanine nucleotides and consist of 3 subunits; α, β and γ. Generally, upon activation of a GPCR by a ligand, conformational changes in the receptor occur that result in the activation of the G protein heterotrimer. Guanosine triphosphate (GTP) replaces guanosine diphosphate (GDP), and the $G\alpha$ subunit and $G\beta\gamma$ dimer dissociate from the receptor and

from one another. It should be noted that this dogma of G protein subunit dissociation has recently been challenged (Bunemann, Frank et al. 2003). The G protein subunits then go on to modulate downstream effectors of signaling cascades that include enzymes like adenylyl cyclase, phospholipases, Src kinases and mitogen-activated protein kinases (MAPKs), as well as calcium and potassium ion channels (Pierce, Premont et al. 2002). Thus, the signal is conveyed from the extracellular ligand to the G protein (the transducer) via the receptor, which results in intracellular signaling.

1.1. Structural classification of GPCRs

From mutagenesis and biochemical approaches, we believe that GPCRs are composed of α-helices that pass the plasma membrane seven times, with the N-terminus of the receptor protein on the extracellular side and the C-terminus on the intracellular side. The membrane-spanning α-helices are arranged in an anti-clockwise fashion and are each composed of about 25-35 amino acids that are mostly hydrophobic. There are technical difficulties in the solubilization and purification of GPCRs for X-ray crystallography (Schioth and Fredriksson 2005). In 2000, Palczewski and coworkers reported a three-dimensional crystal structure of rhodopsin (in its inactive state) (Palczewski, Kumasaka et al. 2000), and for several years it was the only GPCR whose high-resolution crystal structure was available. Recently, a crystal structure for the β_2 adrenergic receptor (β2-AR) has been proposed, with the receptor being occupied by a partial inverse agonist, carazolol (Cherezov, Rosenbaum et al. 2007).

GPCRs are generally classified by structure into 6 classes, with classes A, B, and C being the major ones (Horn, Bettler et al. 2003; Devi 2005).

Class A (rhodopsin-like) is the largest class of the GPCR superfamily. Receptors in this class include those that bind peptides as well as small-molecule ligands, such as biogenic amines, photons, and nucleosides. There are few highly conserved residues amongst class A receptors, namely the transmembrane DRY (helix 3) and NPXXY (helix 7) motifs. Representative class A receptors include receptors for dopamine, histamine, serotonin, epinephrine, norepinephrine, angiotensin, bradykinin, cannabinoids, thyrotropin-releasing hormone and platelet-activating factor. Generally, small-molecule ligands bind within their receptors' transmembrane core, while the larger peptide ligands bind to the extracellular domain.

Class B (secretin-like) includes about 20 receptors for hormones and neuropeptides. These receptors share a large N-terminus, as well as conserved cysteine residues in the N-terminus and extracellular loops that putatively form a network of disulfide bridges. The N-terminus and extracellular loops are the sites of ligand binding to the receptors. The conserved proline residues in this class are different than those of class A receptors. Class B includes the calcitonin, gastric inhibitory peptide, glucagon and parathyroid hormone receptors, among others.

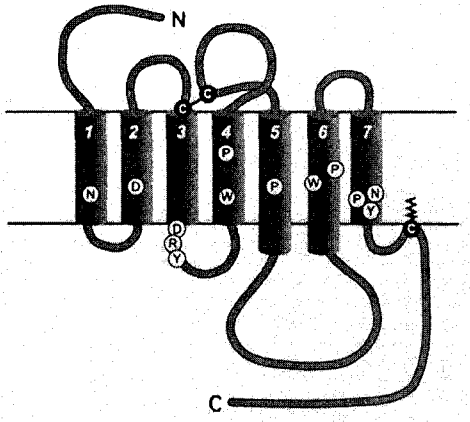
Class C (metabotropic glutamate/pheromone) receptors have a very large "Venus flytrap" N-terminus that binds the ligand. This domain is between 300-600 residues long. Highly conserved between class C receptors is the NEAK motif on the very short, third intracellular loop. Examples of class C GPCRs are the metabotropic glutamate, calcium-sensing-like, pheromone, GABA-B and taste receptors.

The only common features between classes A, B and C receptors are a conserved cysteine residue in helix III and another cysteine residue in the second extracellular loop. These two cysteines presumably form a disulfide bridge important for structural integrity.

The minor classes of GPCRs are the class D fungal pheromone receptors, class E cAMP receptors and the newly-added frizzled/smoothened receptor family.

Figure 1 illustrates key elements in class A, B and C receptors.

Figure 1. Schematic illustrations of the three main classes of GPCRs, with common motifs shown. Black letters in white circles indicate conserved residues for each family. White letters in black circles represent cysteine residues connected by a disulfide bridge. Most class A receptors have a palmitoylated cysteine in the C-terminus that may result in a fourth intracellular loop (Gether 2000).



Family A. Rhodopsin/ß2 adrenergic receptor-like

Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)

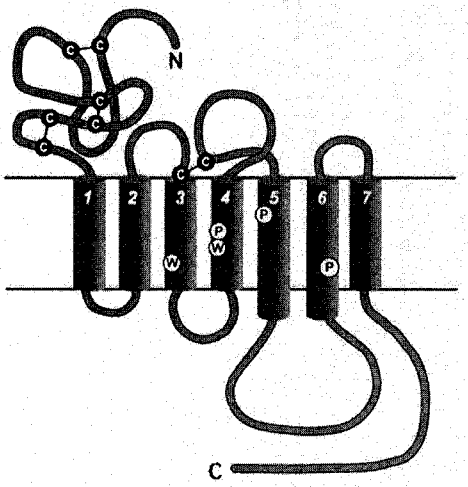
CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins

Invertebrate opsins and bradykinin receptors

Adenosine, cannabinoid, melanocortin, and olfactory receptors.

Chemokine, fMLP, C5A, GnRH, eicosanoid, leukotriene, FSH, LH, TSH, fMLP, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and protease-activated receptors plus others.

Melatonin receptors and other non-classified



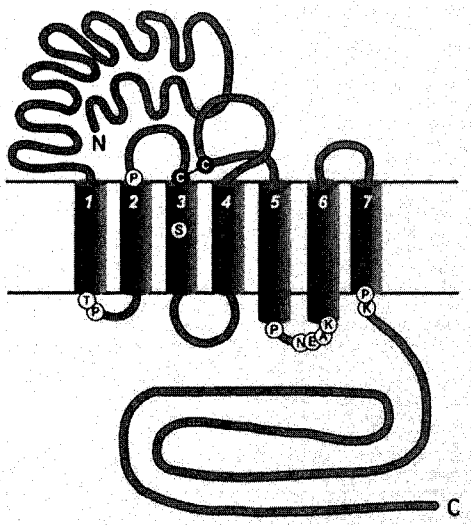
Family B. Glucagon/VIP/Calcitonin receptor-like

Calcitonin, CGRP and CRF receptors

PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GIP, GHRH, PACAP, VIP, and secretin receptors

Latrotoxin



Family C. Metabotropic neurotransmitter/ Calcium receptors

Metabotropic glutamate receptors

Metabotropic GABA receptors

Calcium receptors

Vomeronasal pheromone receptors

Taste receptors

1.2. Ligand binding to and activation of class A receptors

Since the receptor discussed in this thesis is the bradykinin type 2 receptor, a class A GPCR, this section will briefly describe the receptor conformational changes and activation that occur upon ligand binding to class A (rhodopsin-like) GPCRs.

1.2.1. Ligand binding to rhodopsin and biogenic amine receptors

Rhodopsin is a unique receptor in the sense that its endogenous ligand, the photochromophore 11-*cis*-retinal, is covalently bound to the receptor in a crevice formed by the transmembrane helices. Behaving as an inverse agonist, 11-*cis*-retinal keeps the receptor in the inactive state in the absence of light. Upon exposure to light, 11-*cis*-retinal isomerizes to all-*trans*-retinal and the receptor is activated (Gether 2000).

Just as for rhodopsin, receptors for small molecules have a binding crevice situated in the transmembrane area. Examples of such receptors are the dopamine, serotonin, histamine, epinephrine, norepinephrine and acetylcholine receptors. The binding crevice is deeply buried and is outlined by residues in the transmembrane segments (TM) 3, 5, 6 and 7. Of importance is an aspartate residue in TM 3 which is conserved among biogenic amine receptors and is believed to form a salt bridge with positively charged biogenic amine ligands (Gether 2000). However, an exception is the δ-opioid receptor for which this aspartic acid residue was not found to be important for high-affinity ligand binding, but which nevertheless plays a role in stabilizing the spatial conformation of the binding crevice (Befort, Tabbara et al. 1996).

While it is widely acknowledged that small molecules bind within the transmembrane core of class A receptors, it seems that the second extracellular loop (ECL 2) connecting TM 4 and TM 5 may be involved for some ligands. Zhao and coworkers found that for the α_{1a}- adrenergic receptor, the antagonists phentolamine and WB4101 required the

presence of three amino acids located in ECL 2 (Q196, I197, N198), suggesting that antagonists for this receptor bind near the receptor surface, unlike α_1- adrenergic receptor agonists which bind within the TM core (Zhao, Hwa et al. 1996). Shi and Javitch examined the possibility of the involvement of ECL 2 in ligand binding to the D2 dopamine receptor. ECL 2 indeed does seem to be involved for antagonist binding to the D2 dopamine receptor, much like for bovine rhodopsin whose ECL 2 folds down into the binding pocket and forms a lid over retinal (Shi and Javitch 2004).

1.2.2. Ligand binding to class A peptide receptors

Unlike for small molecule-binding receptors, the majority of class A peptide receptors studied were found to bind ligands via the amino terminus and extracellular loops (Gether 2000). For the angiotensin II type 1 receptor (AT1R), regions that are important determinants for binding of the agonist angiotensin II and the peptide antagonist [Sar^1, Leu^8]AII were found to be located around the tops of TM 1, 2 and 7. These regions, which are presumably close to one another in the folded receptor, did not seem to be important for the binding of nonpeptide antagonists to AT1R (Hjorth, Schambye et al. 1994). Both AT1R and the angiotensin II type 2 receptor (AT2R) bind angiotensin II (Ang II), even though the two receptors share only 34% sequence homology. It has been shown that Ang II binds AT1R and AT2R at different contact points but with the same orientation in the receptor binding pocket (Heerding, Yee et al. 1997; Servant, Laporte et al. 1997; Laporte, Boucard et al. 1999; Deraet, Rihakova et al. 2002). Thus, the AT1R and AT2R have evolutionarily diverged in their mechanisms for high-affinity binding of angiotensin II. For the V2 vasopressin receptor, it was found that residues in extracellular loops and the tops of transmembrane regions are important for ligand binding as well as

for determining species differences in binding of the receptor to peptide agonists and antagonists (Cotte, Balestre et al. 1998).

For the bradykinin type 2 receptor (B2R), one study found that in addition to the involvement of residues on the tops of TM 6 and 7, a lysine residue in ECL 2 (Lys^{172} in human B2R) is also involved in binding to bradykinin (BK) (AbdAlla, Jarnagin et al. 1996). This lysine residue seems to be near the site of binding of the N-terminus of the BK agonist, and an analogous residue playing this role was found in the rat B2R (corresponding to Lys^{174}). In fact, antibodies to this extracellular domain not only compete with B2 agonists but are also able to activate the receptor, as seen with a transient increase in intracellular Ca^{2+} (AbdAlla, Quitterer et al. 1996).

However, it seems that in some instances peptide ligands also interact with the transmembrane regions of receptors. An interesting finding for the bradykinin type 1 and 2 receptors (B1R and B2R, respectively) was that a single residue in TM 3 was found to be responsible for ligand selectivity between the two receptor subtypes (Fathy, Mathis et al. 1998). B1R and B2R possess 36% homology and differentiate between ligands solely on the basis of the ligand lacking (for B1R) or having (for B2R) a C-terminal Arg residue. When the afore-mentioned position in TM 3 is occupied by a Lys, as for wild-type B1R (Lys^{118}), the Lys repels the positive charge of the C-terminal Arg in B2-selective peptides and attracts the negative charge of B1-selective peptides (which lack the Arg residue). The wild-type B2R has a Ser corresponding to this position (Ser^{111}) and allows B2-selective peptides to bind. Unlike for B1R, no residues in TM 3 were found in B2R that directly interact with peptide ligands. Also, Ser^{111} is not conserved in the B2R among different species.

1.2.3. Molecular mechanisms involved in GPCR activation

A major point of interest in GPCR research is understanding how the agonist-bound receptor signals; namely, what mechanisms occur to activate the heterotrimeric G protein, the main route through which a GPCR transmits its signal to the inside of a cell. The extracellular portion of the receptor is known to bind ligands, while the intracellular surface is important for G protein activation. One approach to investigate the mechanisms by which a ligand-bound GPCR in turn activates its bound G protein is by looking at highly conserved amino acid motifs in GPCRs. As previously mentioned, class A GPCRs have a conserved E/DRY motif, consisting of the amino acid triplet glutamic acid/aspartic acid-arginine-tyrosine. The E/DRY motif is located on the cytoplasmic side of TM 3 (Rovati, Capra et al. 2007). It appears that the arginine of the E/DRY motif is constrained in a hydrophilic pocket formed by conserved polar amino acids in TM1, TM2 and TM7, and that receptor activation causes protonation of the aspartic acid residue (D) in the DRY motif, resulting in the arginine residue being moved out of the pocket (Scheer, Fanelli et al. 1996; Scheer and Cotecchia 1997). Previously hidden cytoplasmic sequences in the second and third intracellular loops (ICL2 and ICL3), presumably important for G protein recognition and activation, subsequently become exposed to the G protein.

Mutation of the aspartic acid of the DRY motif causes some class A receptors to become constitutively active mutants; that is, receptors with greater agonist independence. This has been found for the α_{1b} adrenergic receptor (Scheer, Fanelli et al. 1996; Scheer and Cotecchia 1997), the β_2 adrenergic receptor (β2-AR) (Rasmussen, Jensen et al. 1999) and other GPCRs.

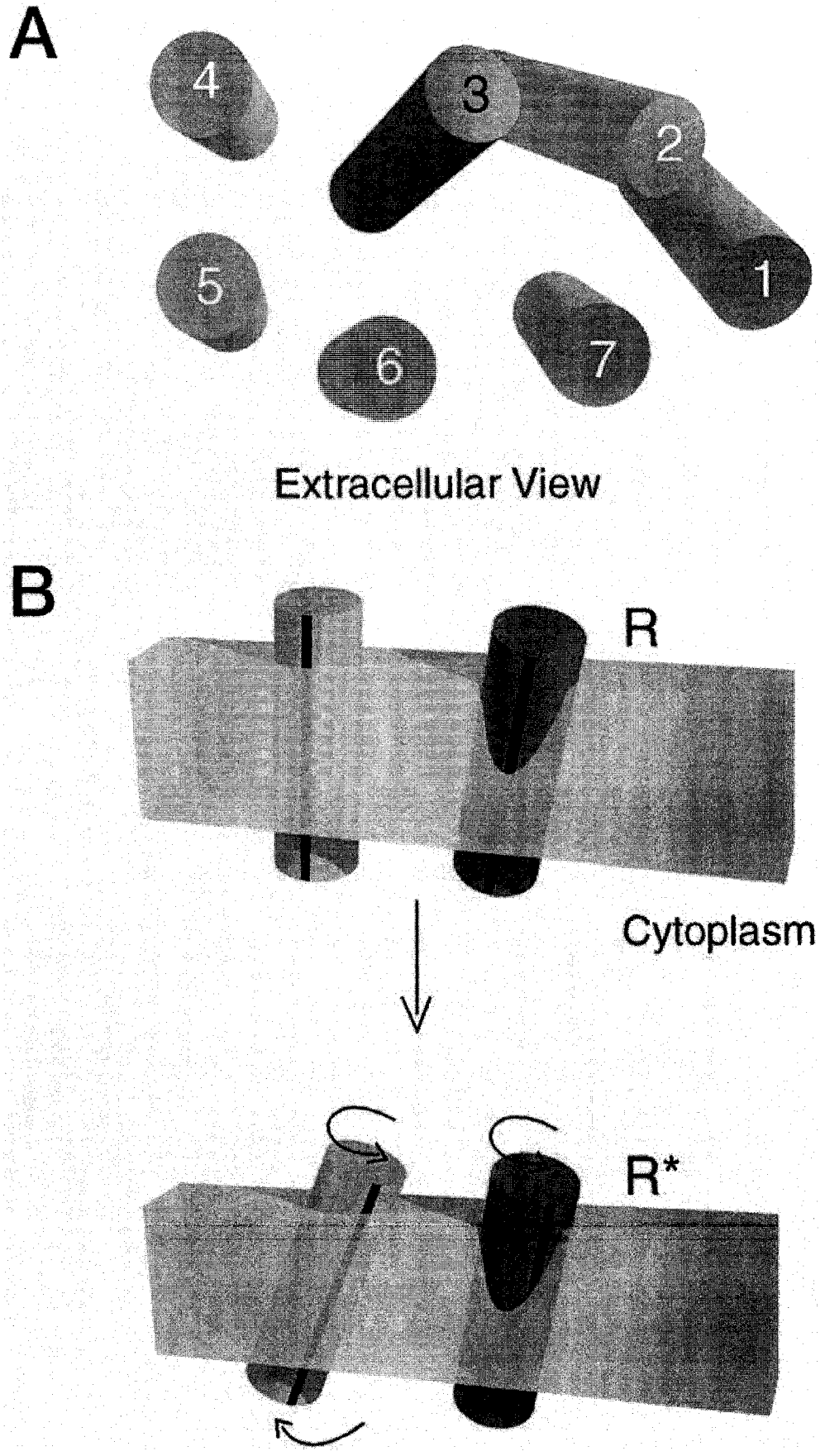
Similarly, mutations in the highly conserved NPxxY motif result in constitutively active mutants. This motif consists of asparagine, proline and tyrosine (the "x" stands for

any amino acid). In the case of the visual receptor rhodopsin, the cytoplasmic region of the NPxxY motif was found to contain binding sites for transducin, rhodopsin's G protein (Fritze, Filipek et al. 2003). Another example is the serotonin 5HT2C receptor, in which mutations of the tyrosine residue in the NPxxY motif to all other naturally occurring amino acids resulted in varying degrees of receptor constitutive activity (Prioleau, Visiers et al. 2002). The authors of the study implicated the conserved tyrosine residue in contributing to the multiple conformational states the 5HT2C receptor undergoes as it is activated.

There are many studies in the literature that have employed mutagenesis to discover other molecular determinants of GPCR activation. Intramolecular interactions help stabilize the receptor in its inactive form, and are disrupted when a receptor is activated. For instance, the C-terminus portion of the third intracellular loop (IC3) was found to be important for keeping the β2-AR inactive. Replacement of this region with a similar region from the α_{1B} receptor caused the β2-AR to become constitutively active (Samama, Cotecchia et al. 1993). As another example, residues in TM3 and TM6 have been found to contribute to the activation of some GPCRs. A study on the human B2 bradykinin receptor found that a tryptophan residue in TM6 (Trp^{256}) is in close proximity to and interacts with an asparagine residue in TM3 (Asp^{113}). This interaction seems to contribute to the stability of the inactive B2 bradykinin receptor (Marie, Richard et al. 2001).

It has been proposed that activation of class A receptors, or at least the β2-AR and rhodopsin, involves movements of TM3 and TM6. Viewed from the extracellular side, both TM segments rotate in a counterclockwise motion; however, TM3's movement is relatively small (Farrens, Altenbach et al. 1996; Gether, Lin et al. 1997). *Figure 2* illustrates this mechanism.

Figure 2. Movement of TM segments upon receptor activation. *A*: arrangement of the transmembrane segments of a class A GPCR, as viewed from the extracellular side, based on crystallization studies of rhodopsin. *B*: the conformational changes thought to occur in TM3 and TM6, based on studies on rhodopsin and the β2-AR. TM3 is shown on the right side and TM6 is on the left. R is the inactive conformation of the receptor and R^* is the conformation that can activate G proteins (Gether and Kobilka 1998).


A
4
3
2
1
5
6
7
Extracellular View
B
R
Cytoplasm
R*

1.3. Heterotrimeric G proteins and their effectors

Ligand-bound GPCRs propagate their signals inside the cell via the activation of heterotrimeric guanine nucleotide-binding proteins (G proteins). The G proteins in turn interact with effectors such as ion channels or enzymes that regulate the production of second messengers (Milligan and Kostenis 2006). For example, in the liver, epinephrine (also known as adrenaline) activates the β2-adrenergic receptor (β2-AR). This receptor is coupled to and activates the $G\alpha_s$ protein, which stimulates the enzyme adenylyl cyclase, resulting in increased production of cyclic adenosine monophosphate (cAMP). cAMP then promotes the breakdown of stored glycogen (glycolysis) and inhibits the synthesis of any further glycogen, leading to increased levels of glucose (Exton 1987).

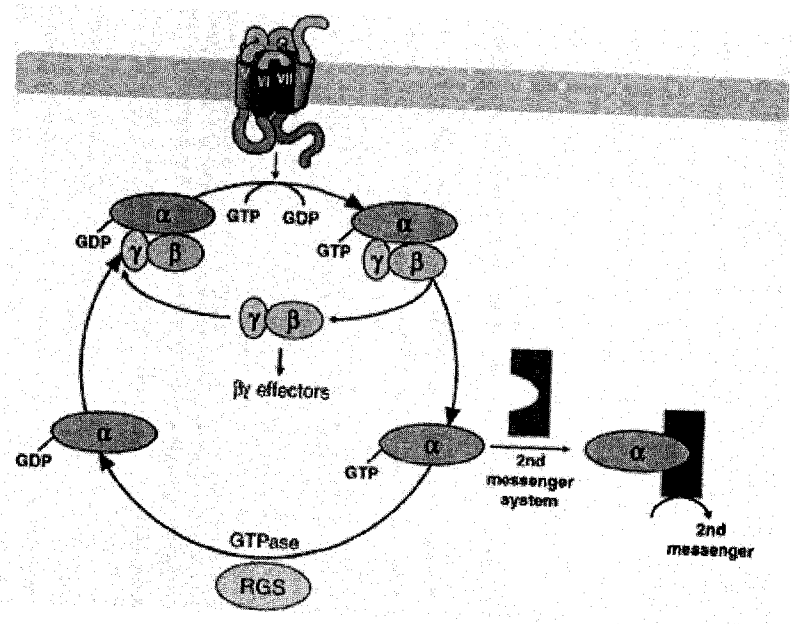
As their name suggests, heterotrimeric G proteins are composed of three different subunits: an α subunit, a β subunit and a γ subunit. In humans, G protein subunits are the products of 35 genes: 16 for α subunits, 5 for β subunits and 14 for γ subunits. The β and γ subunits are considered to be one functional complex. The α subunit can bind guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP), due to the presence of a GTPase domain in the Gα. There are different types of α subunits and they do not all interact with the same effectors. Thus the specific signal generated (or inhibited) depends on the type of subunits coupled to the receptor, but a general model is as follows: the inactive G protein heterotrimer is GDP-bound (via the Gα subunit). When the GPCR it is coupled to is activated, the active receptor behaves as a guanine nucleotide exchange factor (GEF), induces conformational changes in the G protein, and GTP is substituted for GDP on the G protein α subunit. This promotes G protein activation, and now the GTP-bound α subunit dissociates from the βγ dimer. Each of the Gα and Gβγ go on to regulate effectors such as ion channels and second messenger-generating enzymes. Gα, with its

intrinsic GTPase activity, hydrolyzes GTP to GDP, promoting re-association of the heterotrimer and terminating its signaling. The inactivation process is accelerated by regulators of G protein signaling (RGS) which are a family of GTPase-activating proteins (GAP) (Milligan and Kostenis 2006). This cycle of G protein function is summarized in *figure 3*.

GPCRs are described according to which Gα subunits they couple to. For instance, β2-AR is a G_s-coupled receptor. In addition, a GPCR may be promiscuous in its coupling; it is possible for a GPCR to simultaneously couple to different types of G proteins. Studies have shown that this phenomenon does not seem to be an experimental artifact; rather, this is an inherent property of the GPCRs examined. Coupling to different G proteins that give rise to different effector pathways adds a further level of complexity and flexibility to GPCR signaling (Hermans 2003). The β2-AR is known to couple to mainly two types of Gα proteins, $G\alpha_s$ and $G\alpha_i$ (Xiao, Zhang et al. 2003). The bradykinin type 2 receptor (B2R), the subject of the study herein, has been shown to couple to $G\alpha_i$ and $G\alpha_q$ (Liao and Homcy 1993). Further studies are needed in order to better understand the pharmacological and physiological consequences of promiscuous G protein coupling. It appears that for the β2-AR, the $G\alpha_i$ pathway is activated in a negative feedback manner to the $G\alpha_s$ pathway, and that the two pathways play opposite physiological roles (Xiao 2001).

Based on sequence as well as downstream signaling pathways, Gα proteins are grouped into 4 subfamilies: the $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ families. The main members of each family, as well as the effector systems they regulate, will be briefly described in the following sections. Signaling by Gβγ will also be discussed.

Figure 3: General mechanism of heterotrimeric G protein activation and inactivation (Milligan and Kostenis 2006).


VI VII
α
GTP
GDP
GDP
γ
β
α
GTP
γ
β
γ
β
βγ effectors
α
GDP
α
GTP
2nd
messenger
system
α
2nd
messenger
GTPase
RGS

1.3.1. The $G\alpha_s$ family

The $G\alpha_s$ family includes $G\alpha_s$ and $G\alpha_{olf}$. One clue to the identification of the GTP-dependent protein involved in hormonal production of cyclic adenosine monophosphate (cAMP) was the fact that the bacterium responsible for the disease cholera, *Vibrio cholerae*, produced an exotoxin that resulted in sustained and elevated production of cyclic AMP. It was discovered that $G\alpha_s$ becomes constitutively active when exposed to the cholera toxin, an ADP-ribosyl-transferase that covalently attaches ADP-ribose to $G\alpha_s$. Cholera toxin-modified $G\alpha_s$ cannot hydrolyze GTP to GDP and thus remains active (Milligan and Kostenis 2006). Members of this family of G proteins signal via the effector adenylyl cyclase (AC), a plasma membrane-spanning enzyme that catalyzes the production of cAMP from adenosine triphosphate (ATP) (Cooper 2005). Hence the name of this family of G proteins; adenylyl cyclase stimulatory G protein or G_s. Generation of cAMP leads to the activation of protein kinase A (PKA), a ubiquitous serine/threonine kinase consisting of a dimer of regulatory subunits and a dimer of catalytic subunits. PKA becomes activated when two cAMP molecules bind each of the regulatory subunits, resulting in the release of the now active catalytic subunits (Weber, Takio et al. 1982). Some PKA targets include metabolic enzymes, ion channels and transcriptional regulators.

The $G\alpha_{olf}$ protein has been implicated to be involved in olfaction. It seems that when an odorant molecule binds an olphactory GPCR, $G\alpha_{olf}$ is activated, which in turn activates adenylyl cyclase III, increasing cAMP levels. cAMP binds cyclic nucleotide-gated channels, causing them to open and allow the influx of cation ions. This results in an action potential that sends a signal to the brain (Ebrahimi and Chess 1998).

1.3.2. The $G\alpha_{i/o}$ family

Contrary to the $G\alpha_s$ family, the members of the $G\alpha_{i/o}$ family inhibit adenylyl cyclase (the "i" stands for adenylyl cyclase inhibitory). This family includes $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_z$, $G\alpha_{gust}$, $G\alpha_{t1}$ and $G\alpha_{t2}$. With the exception of $G\alpha_z$, the members of this family are sensitive to and inactivated by pertussis, the exotoxin produced by *Bordetella pertussis*, the bacterium that causes whooping cough (Milligan and Kostenis 2006). Pertussis toxin is an ADP-ribosyl-transferase like cholera toxin. However, it inactivates its G protein targets rather than constitutively activate them by targeting cysteine 351 (Avigan, Murtagh et al. 1992). While the G proteins of this family mostly inhibit AC, a lot of their biological effects are mediated by the $\beta\gamma$ subunits they are initially complexed with (Milligan and Kostenis 2006).

$G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ are the products of different genes encoding $G\alpha_i$ members, and they all mediate inhibition of adenylyl cyclases (Itoh, Toyama et al. 1988). On the other hand, $G\alpha_o$ is the product of a gene that can undergo alternative splicing to generate at least two different proteins, $G\alpha_{o1}$ and $G\alpha_{o2}$ (Milligan and Kostenis 2006). $G\alpha_o$ is the most abundant G protein in the mammalian brain and has been shown to be involved in neurite outgrowth (Strittmatter, Fishman et al. 1994). $G\alpha_z$ is still relatively less understood with regards to what receptors couple to it. A recent paper has shown that it is functionally coupled to dopamine D2-like receptors *in vivo* (Leck, Blaha et al. 2006). This G protein is expressed in platelets, neurons, adrenal chromaffin cells and neurosecretory cells. The last three G proteins are involved in sensory responses. $G\alpha_{gust}$ mediates the action of taste receptors, while $G\alpha_{t1}$ and $G\alpha_{t2}$ are found in the retina and are involved in the visual system (Milligan and Kostenis 2006).

1.3.3. The $G\alpha_{q/11}$ family

This family of pertussis- and cholera-insensitive G proteins includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$. $G\alpha_q$ and $G\alpha_{11}$ are ubiquitous in their expression, while the other members are more tissue-specific (Milligan and Kostenis 2006). The members of this family activate phospholipase C-β (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 is a universal calcium-mobilizing second messenger that binds to its receptor in the endoplasmic reticulum, causing Ca^{2+} channels to open and thus increasing intracellular levels of Ca^{2+}. Calcium-sensitive enzymes such as protein kinase C (PKC) are then activated. DAG can also activate PKC (Drin and Scarlata 2007).

There are four known PLC-β family members: PLC-β1, PLC-β2, PLC-β3 and PLC-β4. They are all activated by GTP-bound $G\alpha_q$ family members but in the order β1, β4 > β3 > β2. Gβγ can also activate PLC-β2 and β3 (Drin and Scarlata 2007). *Figure 4* illustrates activation of PLC-β by a GPCR coupled to a G protein from the $G\alpha_{q/11}$ family.

1.3.4. The $G\alpha_{12/13}$ family

This family's two members, $G\alpha_{12}$ and $G\alpha_{13}$, have been shown to be involved in the regulation of cellular shape and morphology as well as cell proliferation. Constitutively active (GTPase-deficient) forms of these G proteins have been linked to stimulation of DNA synthesis, cell proliferation and malignant transformation, as well as the formation of stress fibers and focal adhesions that result in changes in cell shape. The monomeric G protein Rho is often involved in these processes, and is activated by the $G\alpha_{12/13}$ family. This occurs via the interaction of $G\alpha_{12}$ and $G\alpha_{13}$ with a guanine nucleotide exchange factor for Rho (RhoGEF) such as p115-RhoGEF, PDZ-RhoGEF and leukemia-associated RhoGEF (LARG) (Riobo and Manning 2005).

A homology tree of the mammalian G protein α subunits is shown in *figure 5*.

Figure 4: Schematic illustration of the activation of PLC-β, and the generation of IP_3 and DAG, downstream of a $G_{q/11}$-coupled GPCR. *R* is the receptor, *L* is the ligand and *ER* is the endoplasmic reticulum (Drin and Scarlata 2007).

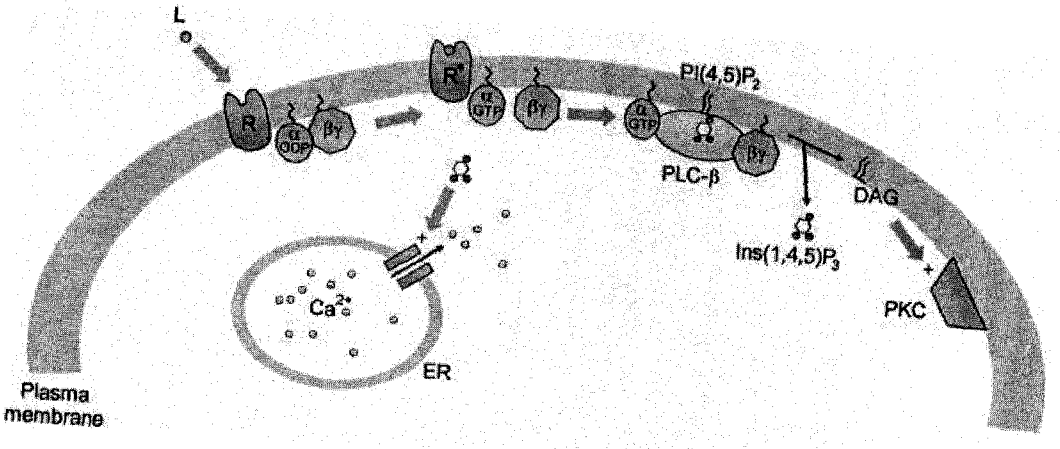
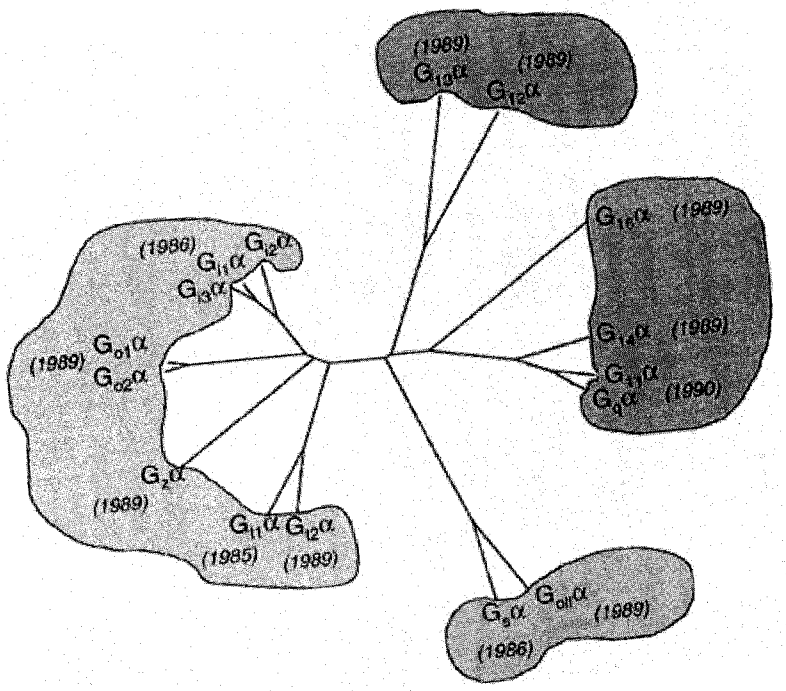

L
R
αGDP
βγ
R*
αGTP
βγ
αGTP
PI(4,5)P2
βγ
PLC-β
DAG
Ins(1,4,5)P3
PKC
Ca2+
ER
Plasma membrane

Figure 5: Homology tree of the mammalian G protein α subunits. Shown next to each member in parantheses is the date its cDNA was cloned (Milligan and Kostenis 2006).


(1989)
$G_{10}\alpha$
(1989)
$G_{12}\alpha$
$G_{13}\alpha$ (1989)
$G_{14}\alpha$ (1989)
$G_{11}\alpha$
$G_q\alpha$ (1990)
(1986)
$G_{i2}\alpha$
$G_{i1}\alpha$
$G_{i3}\alpha$
(1989)
$G_{o1}\alpha$
$G_{o2}\alpha$
$G_z\alpha$
(1989)
$G_{i1}\alpha$ $G_{i2}\alpha$
(1985) (1989)
$G_s\alpha$ $G_{olf}\alpha$ (1989)
(1986)

1.3.5. Gβγ signaling

To date, at least 5 different β and 12 different γ subunits have been reported. The Gβγ dimer, considered a single functional complex, has been shown to participate in signaling downstream of activated GPCRs. It was previously thought to be a passive dimer that serves to bind an activated Gα subunit back to the inactive heterotrimer and to anchor the Gα subunit to the plasma membrane. It took some time for the "βγ signaling hypothesis" to become widely accepted (Milligan and Kostenis 2006). The first evidence that the Gβγ complex can participate in signaling came from a study reported in 1987. The study found that purified βγ subunits are able to activate muscarinic acetylcholine receptor-gated potassium channels in chick embryonic atrial cells (Logothetis, Kurachi et al. 1987). Thus, the G protein-gated inwardly rectifying potassium channel (GIRK) was the first Gβγ effector to be discovered. When GIRK is activated, the potassium (K^+) current increases and heart rate decreases.

Other Gβγ effectors discovered over the years include: PLC-β, some adenylyl cyclases (however at least one, AC-I, is inhibited by Gβγ), calcium channels, phosphoinositide 3-kinase and others. It should be noted that while a number of different β and γ combinations have been observed, there are some that are known not to occur. For example, β_1 has been found to complex with each of γ_1 and γ_2 (as well as all other known γ subunits). β_2, on the other hand, can pair with γ_2 but not γ_1 (Clapham and Neer 1997).

1.3.6. Recent challenges to the dogma of G protein subunit dissociation

As previously mentioned, the classic model of G protein activation states the requirement of Gα subunit dissociation from the Gβγ dimer. However, in more recent years evidence has suggested that this might not necessarily occur, at least for some G protein subtypes. One study examined the mating G protein of the yeast *Saccharomyces*

cerevisiae and found that a fusion protein of the α and β subunits activated the mating pathway as well as when they are coexpressed individually (Klein, Reuveni et al. 2000). Interestingly, a Gα subunit has been shown to have a binding site on the Gβγ effector GIRK. Studies in *Xenopus* oocytes found that $G\alpha_{i3}$ binds to GIRK, inhibiting its basal activity and enhancing its activation by Gβγ (Peleg, Varon et al. 2002), and that $G\alpha_{i3}$ is more efficient in priming GIRK for activation by Gβγ than its relative $G\alpha_{i1}$ (Ivanina, Varon et al. 2004). A proposed mechanism for the action of $G\alpha_{i3}$ is that it forms a complex with both Gβγ and GIRK.

More compelling evidence came from a study that employed fluorescence resonance energy transfer (FRET) and showed that $G\alpha i_1$ does not dissociate from $G\beta_1\gamma_2$ upon activation of α_{2A}-adrenergic receptor with noradrenaline. Furthermore, activation resulted in intramolecular rearrangement in the G protein, bringing the N-terminal region of $G\beta_1\gamma_2$ closer to the α–helical region of $G\alpha i_1$ (Bunemann, Frank et al. 2003). The interactions of $G\beta_1\gamma_2$ with the remaining members of the Gαi/o family were also investigated (in the context of α_{2A}-adrenergic receptor activation). It was found that $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_z$ also do not dissociate from $G\beta_1\gamma_2$. However, $G\alpha_{o1}$ and $G\beta_1\gamma_2$ exhibited a decrease in FRET signal upon receptor activation, reflecting increased distance between the two species and suggesting that $G\alpha_{o1}$ does dissociate from $G\beta_1\gamma_2$. An alternative explanation is that $G\alpha_{o1}$ may have a unique conformational rearrangement that increases subunit distance without physical dissociation (Frank, Thumer et al. 2005). These studies have suggested that the classical paradigm may not apply to all G protein subtypes, conferring a new type of specificity in G protein signaling.

1.4. Regulators of G protein signaling

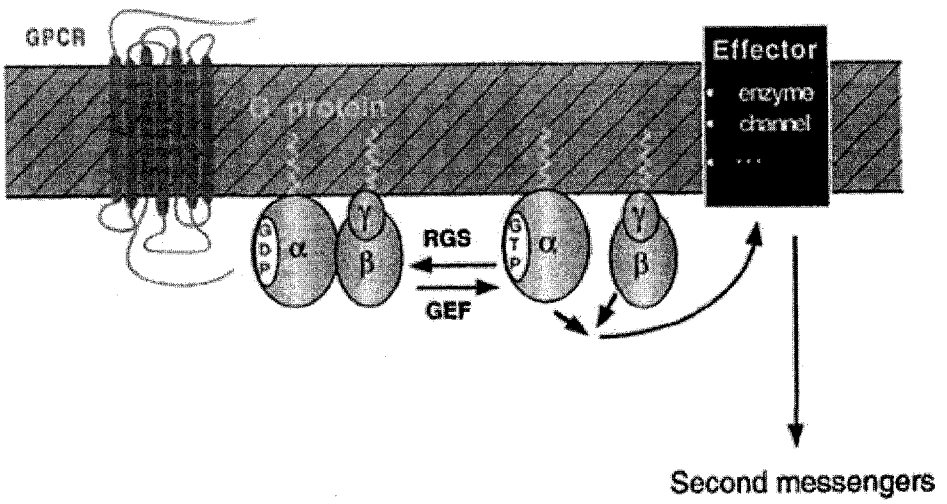
While Gα subunits possess intrinsic GTPase activity, which allows hydrolysis of GTP to GDP and re-formation of the inactive G protein heterotrimer, the rate of hydrolysis is too slow to explain very rapid physiological processes such as vision. Studies on the photoreceptor rhodopsin's G protein transducin showed discrepancies between the fast physiological deactivation of rhodopsin and the slow rate of transducin's GTP hydrolysis observed *in vitro*. This hinted at the presence of GTPase-accelerating proteins that were lost upon purification of transducin for *in vitro* experiments. The family of regulators of G protein signaling (RGS) proteins were eventually shown to be GTPase-activating proteins (GAPs) of Gα subunits. At least 20 mammalian members of the RGS family have been identified thus far, as well as related RGS-like proteins (Ross and Wilkie 2000).

There are three known mechanisms of RGS protein function. First, RGS proteins have GAP activity and thus accelerate the GTP hydrolysis rate of Gα subunits. This GAP activity resides in a conserved RGS domain found in all RGS family members. The RGS domain is also responsible for the binding of the RGS proteins to Gα subunits. The second mechanism is the physical interference of RGS proteins in G protein binding to effectors, interrupting G protein activation of downstream signaling pathways. The third mechanism involves increasing the affinity of Gα for Gβγ and thus favoring the reformation of the G protein heterotrimer (De Vries, Zheng et al. 2000).

It should be noted that some G protein effectors are known to act as GAPs as well, including phospholipase C-β (Berstein, Blank et al. 1992) and adenylyl cyclase type V (Scholich, Mullenix et al. 1999).

Figure 6 illustrates the function of RGS proteins in the regulation of G protein signaling.

Figure 6: Classic model of G protein signaling downstream of an activated GPCR. Once activated, the GPCR acts as a guanine nucleotide exchange factor (GEF) for the G protein, and GTP is substituted for GDP, resulting in G protein activation and dissociation of Gα from Gβγ. The subunits then go on to activate their effectors. RGS proteins serve as GAPs, accelerating the rate of GTP hydrolysis, resulting in Gα becoming GDP-bound again and the inactive G protein heterotrimer reforming (De Vries, Zheng et al. 2000).


GPCR
G protein
Effector
enzyme
channel
...
GDP
α
γ
β
RGS
GEF
GTP
α
γ
β
Second messengers

1.5. G protein-coupled receptor kinases

An important aspect of GPCR function is the attenuation of an activated receptor's signaling. This attenuation mechanism, termed desensitization, protects the cell from the potentially harmful effects of overactive receptor signaling. As previously mentioned, RGS proteins act at the level of the G protein, enhancing the rate of GTP hydrolysis and promoting the reformation of the inactive G protein heterotrimer. The family of G protein-coupled receptor kinases (GRKs) also regulate GPCR signaling, but they do so at the level of the activated receptor itself rather than the G protein. The GRKs are a family of serine/threonine kinases that specifically phosphorylate the active, agonist-occupied forms of GPCRs, and their phosphorylation function prepares the receptor for uncoupling from its G protein (Premont, Inglese et al. 1995).

In humans there are seven GRKs. GRK1 and GRK7 regulate the vision receptors and are expressed exclusively in the retina (with GRK1 found in retinal rods and GRK7 in retinal cones). GRK4 is mostly expressed in the testis, and is also found in the cerebellum and the kidney. GRK2, GRK3, GRK5 and GRK6 are widely expressed. Based on sequence homology, the GRKs are divided into three subfamilies: the rhodopsin kinase subfamily (GRK1 and GRK7), the β-adrenergic receptor kinase subfamily (GRK2 and GRK3) and the GRK4 subfamily (GRK4, GRK5 and GRK6) (Premont and Gainetdinov 2007; Ribas, Penela et al. 2007).

GRKs contain a conserved RGS domain in the N-terminus that is involved in receptor binding, a central catalytic domain responsible for receptor phosphorylation, and a less conserved C-terminus that serves to anchor the GRKs to plasma membrane phospholipids. GRKs also contain sites of binding to various proteins such as clathrin, a component of the clathrin endocytic machinery (the motif is found in the C-terminus

domain of GRKs), phosphoinositide 3-kinase, Gαq/11 (via the RGS domain in the N-terminus) and Gβγ (via the pleckstrin homology domain found in the C-termini of GRK2 and GRK3) (Pao and Benovic 2002).

As previously mentioned, GRKs preferentially phosphorylate agonist-bound GPCRs rather than inactive or antagonist-bound ones. There are no known consensus serine/threonine motifs for GRK-mediated receptor phosphorylation, but it appears that the target serine and threonine residues are found in the third intracellular loop and/or the C-terminus tail. In some cases, the target phosphorylation sites are surrounded by acidic amino acids (Pitcher, Freedman et al. 1998).

Most GRKs are targeted to the plasma membrane by post-translation lipid modifications of their C-termini. GRK1 is farnesylated, GRK4 and GRK6 are palmitoylated, and GRK7 is geranylgeranylated. GRK5 associates with the plasma membrane via its C-terminal phospholipid binding domain, which allows it to bind phospholipids such as PIP_2. On the other hand, GRK2 and GRK3 do not undergo lipid modification, and are instead targeted to the plasma membrane by binding to membrane-anchored Gβγ subunits that have been released from the activated G protein heterotrimer. Their pleckstrin homology domains also allow binding to PIP_2 (Pitcher, Freedman et al. 1998; Penn, Pronin et al. 2000; Kohout and Lefkowitz 2003).

Phosphorylation of an activated GPCR by a GRK primes the receptor for binding to an adaptor protein called arrestin, which physically hinders G protein coupling, resulting in loss of effector activation and signaling. Consequently, the receptor is desensitized.

1.6. The arrestins

Initial studies on GRK2 desensitization of the β2-adrenergic receptor led to a puzzling observation: the greater the purity of the GRK2 preparations used for experiments, the less efficient was the uncoupling of β2-AR from its G_s protein. Adding arrestin, a 48 kDa protein derived from the retina, which was previously discovered to uncouple rhodopsin from its G protein transducin, restored the desensitization of the β2-AR. It seemed highly likely that related proteins exist to regulate the signaling of non-visual GPCRs such as the β2-AR (and that these proteins were lost in the purification of GRK2 for these experiments) (Benovic, Kuhn et al. 1987). This has proven to be true, and there are currently four known members of the arrestin family: visual arrestin and cone arrestin (which are expressed almost exclusively in the retina, where they regulate photoreceptor function), and β-arrestin 1 and β-arrestin 2 (which are ubiquitously expressed in tissues). The arrestins bind to activated GPCRs that have been phosphorylated by GRKs. This results in the receptor-G protein interaction being blocked and the desensitization of the receptor, a process that begins within seconds of receptor activation by agonist. As their name suggests, the arrestins "arrest" GPCR signaling, and this involves three processes: 1. desensitization, 2. sequestration (internalization), and 3. downregulation (Luttrell and Lefkowitz 2002).

1.6.1. Arrestin desensitization of GPCRs

GRKs and arrestins participate in *homologous* desensitization of GPCRs, as opposed to *heterologous* desensitization that may be performed by protein kinases such as PKA and PKC. In the latter type, receptors that are not ligand-bound may be phosphorylated by such protein kinases and be desensitized. These protein kinases would have been activated downstream of other receptors that were bound by agonist; hence the term

heterologous because desensitization can happen on a receptor other than the one being activated. On the other hand, *homologous* desensitization is performed on the same receptor that is activated, and it involves the phosphorylation of the agonist-bound receptor by GRK, followed by arrestin binding to the receptor. This results in the uncoupling of the receptor and the G protein (Chuang, Iacovelli et al. 1996).

The arrestins contain a polar core that resides between its N- and C-terminal domains (see *figure 7*). This polar core serves as a phosphate sensor and is involved in the conformational changes that occur in arrestin upon encountering a phosphorylated receptor, resulting in high affinity binding of arrestin to the GPCR (Han, Gurevich et al. 2001).

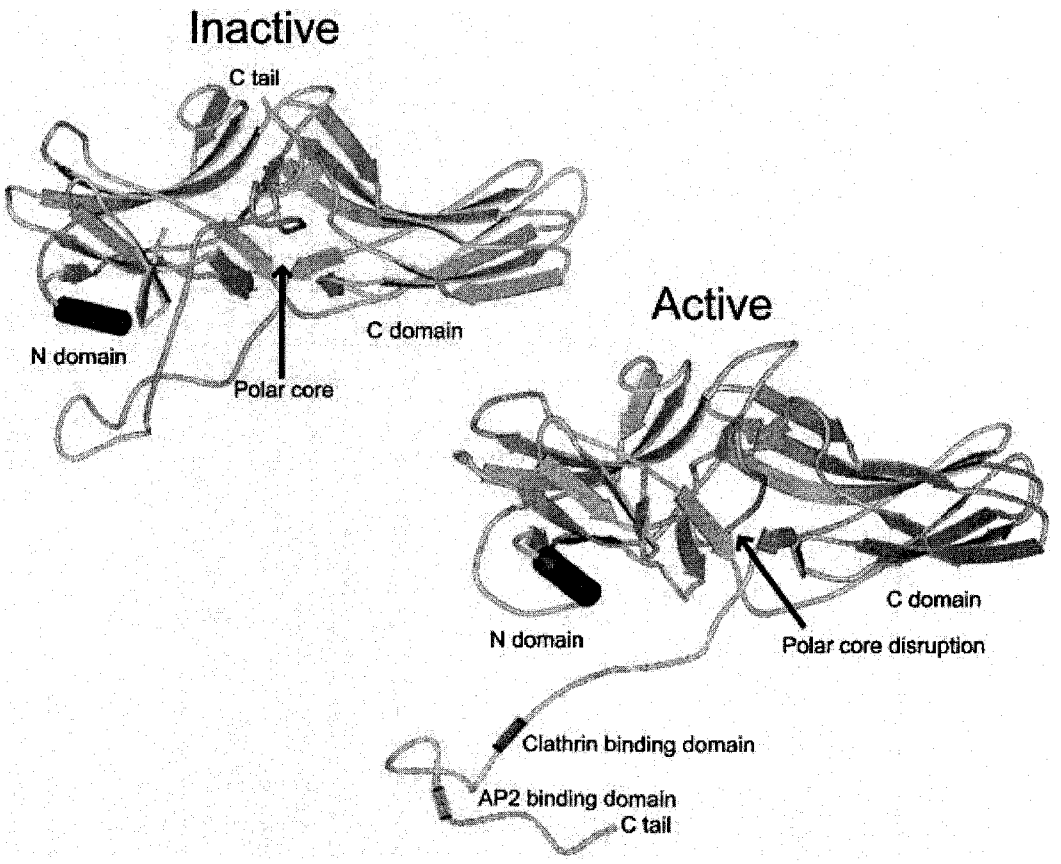
1.6.2. The β-arrestins in GPCR sequestration

1.6.2.1. The mechanisms of clathrin-mediated endocytosis

The β-arrestins perform additional functions that the visual arrestins do not, and one of them is the process of receptor sequestration (also known as internalization or endocytosis). GPCR internalization is a slower process than desensitization, occurring minutes after agonist stimulation, and it serves to clear the receptors away from the plasma membrane and into the cell. The C-terminus of β-arrestin contains binding motifs for clathrin and the β_2-adaptin subunit of AP-2, components of the clathrin-mediated endocytic machinery (see *figure 7*). Studies on the β2-AR showed that β-arrestin can bind the heavy chain of the clathrin triskelion (Goodman, Krupnick et al. 1996; Krupnick, Goodman et al. 1997), and that it can also bind β2-adaptin, a subunit of the AP-2 heterotetramer (Laporte, Oakley et al. 1999; Laporte, Oakley et al. 2000; Laporte, Miller et al. 2002), explaining the targeting of the receptor to clathrin-coated vesicles for endocytosis. Thus, the β-arrestins not only serve to uncouple GPCRs from their G

proteins, but also act as adapters between GPCRs and components of clathrin-dependent endocytosis. *Figure 7* illustrates models of the inactive and active conformations of β-arrestin 2.

Figure 7: Inactive and active conformations of β-arrestin 2. In its inactive state, the polar core of β-arrestin 2 is at the junction of the N- and C-terminal domains. The C-terminal tail is within proximity. Upon interaction of β-arrestin 2 with the phosphorylated tail of an activated receptor, the polar core is disrupted and conformational changes occur, releasing the C-terminal tail. The binding sites for clathrin and AP-2 are now exposed (Lefkowitz, Rajagopal et al. 2006).


Inactive
C tail
N domain
Polar core
C domain
Active
N domain
Polar core disruption
C domain
Clathrin binding domain
AP2 binding domain
C tail

It appears that the majority of GPCRs employ this β-arrestin-mediated, clathrin-dependent mode of internalization. It is possible, however, for GPCRs to internalize by alternative means, via caveolae and other uncoated vesicles (Reiter and Lefkowitz 2006).

The process of clathrin-dependent endocytosis is briefly as follows: the clathrin adaptor protein, AP-2, is found in tight association with clathrin and is required for the formation of clathrin-coated pits at the plasma membrane. With its afore-mentioned adapter function, β-arrestin can recruit a desensitized receptor to clathrin-coated pits via binding to clathrin and the β_2-adaptin subunit of AP-2. AP-2 is a heterotetrameric complex consisting of four subunits: the large subunits α and β_2 (which form the "ear" lobes of AP-2), the medium-size subunit μ_2, and the small subunit σ_2. Each of the four AP-2 subunits have specific functions in the process of endocytosis. Dynamin, a large GTPase, binds the α subunit of AP-2 and pinches off the vesicle. The clathrin-coated vesicle, containing the receptor cargo, is internalized in the cell (Ferguson 2001).

The β-arrestins can also bind other proteins that are involved in the regulation of endocytosis. In the case of β2-adrenergic receptor endocytosis, β-arrestin 1 was shown to recruit c-Src, a non-receptor tyrosine kinase that has been shown to phosphorylate dynamin, a step that is apparently needed for efficient dynamin function (Claing, Laporte et al. 2002). Also, c-Src has been shown to regulate the interaction between β-arrestin and the β_2-adaptin subunit of AP-2 upon stimulation of the AT1R, resulting in c-Src phosphorylation of β_2-adaptin and subsequently, dissociation of the beta-arrestin/AP-2 complex (Fessart, Simaan et al. 2005; Fessart, Simaan et al. 2007). β-arrestin 1 has also been shown to bind to *N*-ethylmaleimide-sensitive fusion (NSF) protein, an ATPase that is essential for vesicle fusion. In addition, β-arrestin has been reported to bind small GTPases such as ADP-ribosylation factor 6 (ARF6). This monomeric G protein is

activated by GEFs such as the ARF nucleotide binding site opener (ARNO), which can also bind β-arrestin, resulting in ARF6 recruitment of clathrin-coat proteins, modification of membrane lipids, and reorganization of the actin cytoskeleton. ARF6 mutants have been shown to reduce internalization of the β2-AR (Claing, Laporte et al. 2002).

A C-terminal serine residue (Ser-412) of β-arrestin 1 has been shown to be phosphorylated, and that this is a regulatory mechanism of the endocytic function of β-arrestin 1 (but not its desensitizing function). Cytosolic β-arrestin 1 is found to be constitutively phosphorylated at this residue. Upon activation of GPCRs and the recruitment of β-arrestin 1, dephosphorylation rapidly occurs. Dephosphorylation is seemingly required for the binding of β-arrestin 1 to clathrin and for subsequent endocytosis. Upon internalization, β-arrestin 1 is phosphorylated again (Lin, Krueger et al. 1997).

1.6.2.2. Receptor internalization dynamics as a function of β-arrestin avidity

There is great homology between β-arrestin 1 and β-arrestin 2; the two isoforms are 78% identical in sequence (Attramadal, Arriza et al. 1992). However, studies have revealed that the β-arrestins have distinct patterns of interaction with GPCRs. Based on their affinities towards arrestins and the stability of the interactions that ensue, GPCRs have been divided into two classes. Members of class A, which includes the β2-AR, the α1B-AR, dopamine D1A, the μ-opioid and the endothelin type A receptors, do not bind visual arrestin and show preference to β-arrestin 2 over β-arrestin 1. Upon binding of β-arrestin to these receptors and their recruitment to clathrin-coated pits, β-arrestin dissociates from the receptors after they are internalized and recycles back to the plasma membrane, whereas the receptors enter the endocytic pathway. Class B receptors, on the other hand, display a receptor- β-arrestin interaction that is not transient. The β-arrestins

remain in complex with these receptors upon internalization, and are targeted to endosomes with them. Members of this class include the angiotensin II type 1A (AT_{1A}), neurokinin-1, vasopressin V2, neurotensin 1, oxytocin and thyrotropin-releasing hormone receptors. Class B receptors have no preference between β-arrestin 1 and 2, and can bind visual arrestin as well. Switching the C-terminus tails of some receptors that were examined changed a class A receptor's behavior to that of a class B receptor, and vice versa (Zhang, Barak et al. 1999; Oakley, Laporte et al. 2000). It has been proposed that the receptor C-terminus has molecular determinants (in the form of clusters of serine and threonine residues) that dictate the stability of the receptor's interaction with β-arrestin. Mutations of these putative phosphorylation sites (serine/threonine to alanine) in some candidate class B receptors resulted in the endocytosis of the receptors, but with β-arrestin 2 remaining at the plasma membrane. Thus, by mutating some of the serine/threonine clusters found in these receptors' C-termini, the receptors lost their ability to stably associate with β-arrestin 2, and internalized without it (Oakley, Laporte et al. 2001).

It should be noted that β-arrestins 1 and 2 have very different cellular distributions in the absence of agonist stimulation. In unstimulated cells, β-arrestin 1 is found in both the cytoplasm and nucleus, whereas β-arrestin 2 is cytosolic only (Oakley, Laporte et al. 2000). This difference in cellular distribution is due to a leucine-rich nuclear export signal (L395/L397) in the C-terminus of β-arrestin 2, while β-arrestin 1 lacks this export signal and is thus found in both the nucleus and cytoplasm in steady-state conditions (Scott, Le Rouzic et al. 2002).

Recent work in our laboratory characterized the internalization, recycling and resensitization of the bradykinin type 2 receptor (B2R). Upon stimulation with bradykinin, B2R is rapidly activated, desensitized and internalized (with internalization

becoming apparent within 5 minutes of agonist stimulation). However, it was previously unknown how B2R is regulated by β-arrestin. Using confocal microscopy, it was shown that B2R does indeed colocalize with β-arrestin 2 into early and/or recycling endosomes. When stimulated cells were washed free of agonist and allowed to recover, the B2R was able to return to the plasma membrane, ready to be re-challenged with agonist. The B2R-β-arrestin 2 complex dissociated, with β-arrestin 2 returning to cytoplasmic distribution. On the other hand, when the C-terminus tail from the vasopressin V2 receptor (a class B GPCR) was substituted for the B2R's tail, the B2R-V2CT chimera remained colocalized with β-arrestin 2 in endosomes, and the complex did not dissociate upon agonist removal. The B2R-V2CT mutant did not recycle back to the plasma membrane (Simaan, Bedard-Goulet et al. 2005). In conclusion, the B2R was found to readily internalize into endosomes along with β-arrestin 2, just like class B receptors. Unlike class B receptors, however, the B2R is able to dissociate from β-arrestin 2 and to rapidly recycle back to the plasma membrane.

The neurokinin type 1 (NK1) receptor, a member of the kinin family that B2R belongs to, also displays stable interaction with β-arrestin, as well as the ability to eventually return to the cell surface (Garland, Grady et al. 1996; McConalogue, Dery et al. 1999). Thus, evidence from our laboratory and others suggests that B2R and NK1R belong to class C, a novel class of GPCRs with trafficking and β-arrestin-binding properties intermediate to those of classes A and B.

1.6.2.3. Implications of unregulated GPCR desensitization and internalization

As a final note on the sequestration process of GPCRs, familial nephrogenic diabetes insipidus illustrates the the physiological importance of properly regulating β-arrestin-dependent desensitization and endocytosis. This condition is commonly caused by

mutations in the vasopressin V2 receptor (V2R), leading to loss of V2R response to its agonist, arginine vasopressin, in the renal tubules. As a result, the kidney is unable to concentrate urine, leading to overexcretion of water, causing dehydration and other symptoms. One of these naturally occurring mutations is a mutation of arginine 137 (of the DRY motif in V2R) to histidine, resulting in V2R being constitutively phosphorylated and bound to β-arrestin. In this case, nephrogenic diabetes insipidus arises from the mutated receptor being constitutively desensitized and trapped in intracellular vesicles along with β-arrestin, even in the absence of agonist (Barak, Oakley et al. 2001). Hence, unregulation of desensitization may lead to pathophysiology, and it is vital that the processes of desensitization and sequestration are properly controlled.

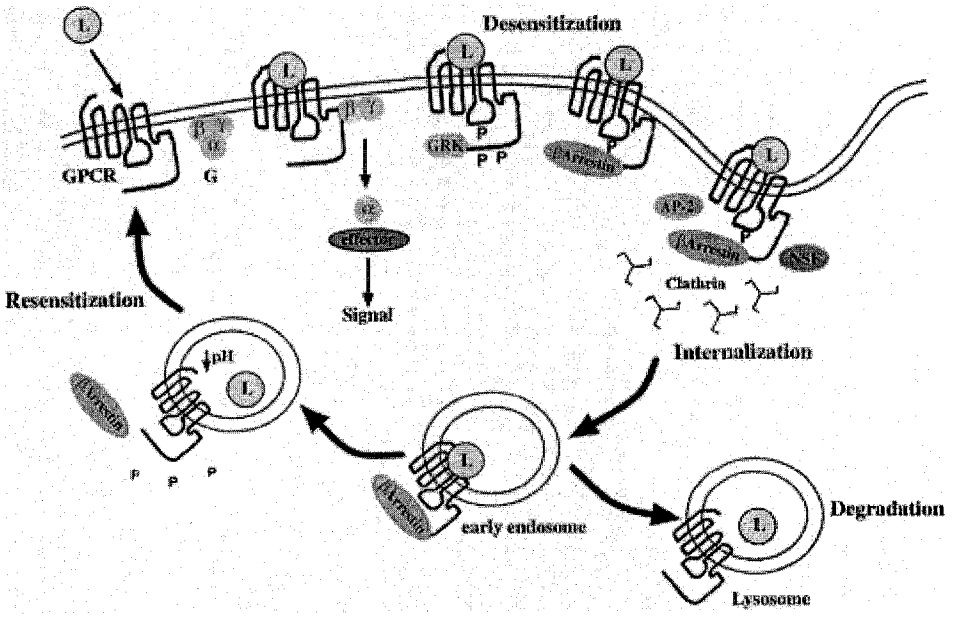
1.6.3. The β-arrestins in the degradation and recycling of GPCRs

Once internalized, GPCRs are either recycled back to the plasma membrane (a process that requires receptor dephosphorylation) and are then ready to be re-activated, or are degraded in specialized vesicles such as lysosomes (Claing, Laporte et al. 2002; von Zastrow 2003). Generally, the fate of the receptor seems to reside in the same C-terminal molecular determinants that regulate the stability of binding to β-arrestin. Receptors that display transient affinity towards β-arrestin (class A receptors) are dephosphorylated by phosphatases in acidic vesicles and rapidly recycled, whereas those receptors that stably interact with β-arrestin via serine/threonine clusters in their C-termini are retained in large endosomes or sent to lysosomes for degradation (Krueger, Daaka et al. 1997; Oakley, Laporte et al. 1999; Anborgh, Seachrist et al. 2000). *Figure 8* illustrates these concepts.

A study by Kohout et al. employed mouse embryonic fibroblast cell lines that were knockouts for β-arrestin 1 and/or β-arrestin 2 in order to examine the desensitization and trafficking of the β2-AR and $AT_{1A}R$. Knocking out either β-arrestin 1 or β-arrestin 2

impaired the desensitization of the receptors, and knocking out both β-arrestins caused even further impairment. As for receptor endocytosis, the β2-AR was affected in its internalization only when β-arrestin 2 or both β-arrestin 1 and β-arrestin 2 were knocked out. The internalization of $AT_{1A}R$, on the other hand, was only significant when both β-arrestins were knocked out. These results confirmed previous studies that showed that the internalization of β2-AR (a class A receptor) mostly involves β-arrestin 2, while the internalization of $AT_{1A}R$ (a class B receptor) may occur with either β-arrestin (Kohout, Lin et al. 2001).

Figure 8: Fate of a GPCR following its activation. A GPCR is activated by ligand (*L*), and its signaling is desensitized when it is phosphorylated by a GRK and then bound by β-arrestin. The receptor is internalized in clathrin-coated vesicles, a process that is β-arrestin-dependent. The vesicles lose their clathrin coat and become early endosomes. Receptors that display transient interaction with β-arrestin, such as the β2-AR, are trafficked to acidified compartments, where they are dephosphorylated by phosphatases and then recycled back to the plasma membrane, ready to be resensitized by ligand. Receptors that stably interact and internalize with β-arrestin, such as the $AT_{1A}R$, are either retained in large endosomes and/or are trafficked to lysosomes for degradation (McDonald and Lefkowitz 2001).


Desensitization
GPCR
G
GRK
P P
Arrestin
AP-2
Clathrin
NSF
Internalization
Signal
Resensitization
pH
early endosome
Degradation
Lysosome

1.6.4. The β-arrestins as scaffolds for signaling

In more recent years, alternative functions have emerged for β-arrestins besides the regulation of homologous desensitization and trafficking of GPCRs. It has been shown that β-arrestins can participate in a "second wave" of signaling, downstream of desensitized GPCRs, by acting as scaffolds or adaptors for signaling proteins. The list of signaling proteins that can interact with β-arrestins keeps on growing, as more and more attention is turned towards understanding this novel role of β-arrestins. These exciting findings have challenged the classical paradigm that GPCRs signal solely via their G proteins, and they highlight the flexibility and complexity of GPCR functions. Some of the proteins whose signaling is mediated by the β-arrestins are:

1. The Src family of tyrosine kinases

Some of the earliest evidence of the involvement of β-arrestins in signaling came from studies on members of the Src family of non-receptor tyrosine kinases. Upon activation of the β2-AR, β-arrestin 1 forms a complex with c-Src and this complex colocalizes with the receptor in clathrin-coated pits. It was found that c-Src binds the N-terminus of β-arrestin 1 via its Src homology (SH) 3 domain, which recognizes Pro-X-X-Pro as its minimal consensus sequence (where X is any amino acid) (Luttrell, Ferguson et al. 1999). It was also found that SH1, the catalytic domain of c-Src, also participates in binding to the N-terminus of β-arrestin 1 (Miller, Maudsley et al. 2000). One outcome of the recruitment of c-Src by β-arrestin is the phosphorylation of the large GTPase dynamin on its tyrosine residues at positions 231 and 597. Mutations of these target residues resulted in inhibition of β2-AR internalization, and it was concluded that tyrosine

phosphorylation of dynamin by c-Src is essential for β2-AR endocytosis (Ahn, Maudsley et al. 1999).

GRK2 was also discovered to be a target of c-Src phosphorylation in a β-arrestin-mediated manner. Activation of β2-AR or the chemokine receptor CXCR4 results in the phosphorylation of GRK2 by c-Src, leading to ubiquitination of GRK2 and its proteosomal degradation. This effect was lost when a β-arrestin 1 mutant with impaired binding to c-Src was used (Penela, Elorza et al. 2001).

Other proteins have been found to bind c-Src (and other members of the Src family) upon β-arrestin recruitment of Src. They include the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinases (MAPKs) (Luttrell, Ferguson et al. 1999), and it turned out that the ERK1/2 MAPKs can directly interact with β-arrestins as well.

2. The mitogen-activated protein kinase (MAPK) family

The MAPKs are a family of ubiquitous serine/threonine kinases that regulate processes such as embryogenesis, cell proliferation, cell differentiation and cell death. They are regulated by a sequential phosphorylation cascade: an upstream MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK), which then phosphorylates and activates MAPK. The MAPKKs found so far belong to the MAP/ERK kinase (MEK) family (Pearson, Robinson et al. 2001).

MAPK pathways are regulated by scaffolding proteins, which serve the following functions: 1) increasing the efficiency of the MAPK phosphorylation cascade, 2) increasing specificity and minimizing interference between parallel MAPK cascades, and 3) localizing the MAPK cascades to specific cellular locations (Pearson, Robinson et al.

2001). The β-arrestins have been proposed to act as scaffolds for GPCR-mediated MAPK activation.

One MAPK cascade is the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK). The JNK/SAPKs are mostly activated by cytokines, some GPCR ligands, agents that disrupt protein and DNA synthesis, and other stresses (Pearson, Robinson et al. 2001). The neuronal JNK/SAPK, JNK3, was found to bind β-arrestin 2. Expressing JNK3 with its MAPKKK ASK1 in COS-7 cells did not result in significant JNK3 activation unless β-arrestin 2 was also co-expressed. Stimulation of the angiotensin II type 1a receptor (AT1aR) resulted in JNK3 activation and the colocalization of JNK3 and β-arrestin 2 inside the cell. ASK1 was found to bind to the N-terminus of β-arrestin 2, and JNK3 to its C-terminus. β-arrestin 1 did not display avid interaction with JNK3. The MAPKK MKK4 was found to interact indirectly with β-arrestin 2, via JNK3 and/or ASK1 (McDonald, Chow et al. 2000; Miller, McDonald et al. 2001).

The members of the p38 subfamily of MAPKs are activated by cytokines, hormones, GPCRs, osmotic and heat shocks, as well as other stresses (Pearson, Robinson et al. 2001). β-arrestin 2 is involved in the chemotactic function of the chemokine receptor CXCR4. It was found that β-arrestin 2 mediates this function via the p38 MAPK because inhibiting p38 or using mutants of p38 or Ask1 (a MAPKKK of p38) blocked the chemotaxis mediation by β-arrestin 2 (Sun, Cheng et al. 2002).

ERK1 and ERK2 belong to a third subfamily of MAPKs and share 85% sequence identity. They are proteins of 43 kDa and 41 kDa, respectively. These proteins are ubiquitously expressed and are each phosphorylated on two target residues (a tyrosine and a threonine) in order to be activated. Their stimulants may differ according to cell type, but in fibroblasts they are activated by serum, growth factors, cytokines, GPCRs and

certain stresses. ERK1 and ERK2 are both activated by the MAPKKs MEK1 and MEK2. Dual phosphorylation (ie, phosphorylation of both the tyrosine and threonine target sites) results in a great increase in ERK1/2 activation, although phosphorylation of either one of the sites is also sufficient. MEKs are activated by members of the Raf family, most notably Raf-1, found upstream of MEKs in the ERK cascade. Raf-1 in turn is activated by H-, N- and K-Ras. Protein kinases such as Src and PKCs can enhance activation of Raf-1 by phosphorylating it (Pearson, Robinson et al. 2001). Activated ERKs go on to activate cytosolic substrates and/or undergo sustained (in the case of mitogenic signals) or transient retention in the nucleus, where they regulate gene transcription (Caunt, Finch et al. 2006).

The proteinase-activated receptor 2 (PAR2) was found to form a complex with β-arrestin 1, Raf-1, and ERK1/2 upon agonist stimulation. Expression of a dominant negative β-arrestin inhibited PAR2 activation of ERK (DeFea, Zalevsky et al. 2000). Similarly, activation of $AT_{1A}R$ was shown to form a complex of $AT_{1A}R$, β-arrestin 2, Raf-1, MEK1 and ERK2 (Luttrell, Roudabush et al. 2001). The β-arrestin-ERK complexes were found to be relatively stable in these studies.

In one study, expressing β-arrestin 1 or β-arrestin 2 decreased the angiotensin-mediated phosphatidylinositol hydrolysis by $AT_{1A}R$, while enhancing ERK1/2 phosphorylation and activation, an apparently paradoxical observation. An increase in the cytosolic pools and a decrease in the nuclear pools of ERK1/2 was observed, suggesting that ERKs activated by the β-arrestin scaffold are retained in the cytoplasm (Tohgo, Pierce et al. 2002). Depletion of β-arrestin 1 and β-arrestin 2 with siRNA resulted in a great loss of $AT_{1A}R$ internalization and ERK1/2 activation (Ahn, Nelson et al. 2003). It has been proposed that the stability of a receptor's binding to β-arrestin dictates the

spatial distribution and physiological functions of activated ERK1/2. Class B receptors, such as $AT_{1A}R$ and V2R, activate β-arrestin-bound ERK2 more readily than the class A receptors β2-AR and α_{1b}-AR, which bind β-arrestin transiently. The C-terminal tails of V2R and β2-AR were exchanged, converting a class A receptor to a class B receptor, and vice versa, and the patterns of ERK activation were correspondingly reversed. V2R was found to activate a greater cytoplasmic pool and lesser nuclear pool of ERK1/2 compared to V2R with a β2-AR tail. The V2- β2 tail chimera displayed greater expression of an Elk1-luciferase reporter (Elk1 is a nuclear transcription factor) (Tohgo, Choy et al. 2003).

A more recent study sought to delineate the two separate, but overlapping, pathways of ERK1/2 activation by the $AT_{1A}R$: the G protein-dependent phase (most likely via $G_{q/11}$) and the β-arrestin-dependent phase. Tools such as RNA interference (to greatly reduce β-arrestin expression) and an inhibitor of PKC (since PKC is one effector of the $G_{q/11}$ pathway) were employed. Distinct patterns of temporal and spatial activation of ERK1/2 were described: the G protein-dependent phase was found to be rapid in onset and transient (having a half-life of about 2 minutes), with the activated ERK1/2 translocating to the nucleus. On the other hand, the β-arrestin-dependent phase maximally activated ERK1/2 at a later time point than the G protein-dependent phase (between 5-10 minutes) and the signal persisted for at least 90 minutes. In this case, the activated ERK1/2 remained localized in the cytoplasm in the same vesicles containing the $AT_{1A}R$-β-arrestin complexes (Ahn, Shenoy et al. 2004).

It should be noted that GPCRs can also activate ERKs in a β-arrestin-independent manner by crosstalk with the tyrosine kinase epidermal growth factor receptor (EGFR). Transactivation of EGFR by GPCRs leads to activation of Ras, and subsequently, activation of the ERK cascade (Gschwind, Zwick et al. 2001).

Some functional consequences of ERK1/2 activation by β-arrestins are: cell migration and pseudopodial extension upon PAR2 activation; nonapoptotic programmed cell death upon NK1R activation; and cell differentiation via activation of tropomyosin-related kinase receptor (Shenoy and Lefkowitz 2005).

3. The E3 ubiquitin ligase Mdm2

Ubiquitination is a post-translational modification involving the covalent addition of ubiquitin, a low-molecular weight protein, to lysine side chains in target proteins. Three enzymes act together: E1 (ubiquitin-activating enzyme), E2 (ubiquitin carrier protein) and E3 (ubiquitin protein ligase). When proteins are polyubiquitinated, they are targeted for degradation by a multisubunit protease complex called the 26S proteasome (Hershko and Ciechanover 1998). It has become apparent that β-arrestin is a target of Mdm2, an E3-ubiquitin ligase. Mdm2-mediated ubiquitination of β-arrestin seems to be essential for receptor internalization, since it serves as a signal for the proper sorting of endocytic cargo. It appears that the kinetics of de-ubiquitination mirror the avidity of the receptor-β-arrestin interaction, since transient ubiquitination results in transient receptor-β-arrestin binding, whereas stable ubiquitination leads to stable receptor-β-arrestin binding. In fact, a class A receptor can be converted to a class B receptor with the use of a β-arrestin 2-ubiquitin chimera and is retained longer in endocytic vesicles, leading to receptor degradation (Shenoy and Lefkowitz 2003).

The β-arrestin-interacting proteins described above (the Src family of tyrosine kinases, the MAPKs and Mdm2) are by no means an exhaustive list. More and more proteins are being discovered to bind β-arrestin and are being added to the repertory of

proteins that scaffold on β-arrestin, with research aiming to elucidate the signaling consequences of such interactions (DeWire, Ahn et al. 2007).

1.7. The physiological actions of the kallikrein-kinin system

Bradykinin (BK) is a vasoactive peptide belonging to the kallikrein-kinin system (KKS). Kinins (BK and lys-BK) cause vasodilation and natriuresis and are well known to contribute to lower blood pressure (Regoli and Barabe 1980; Madeddu, Emanueli et al. 2007). They antagonize the actions of the renin-angiotensin system, which is vasoconstrictory. Since arterial hypertension is postuled to arise from the overactivation of vasoconstrictors and the suppression of vasodilators, it is important to further our understanding of how these two systems interplay. The KKS includes the kallikreins (kinin-forming enzymes), kininogens (the substrates of kallikreins), kinins (vasoactive peptides), kinin-degrading enzymes and kinin receptors. The kallikrein hK1 is involved in the formation of bradykinin and is distributed in the kidney, pancreas, salivary glands, brain, leukocytes and cardiovascular tissue. In circulation, kinins are subject to degradation by kininases; hence their action is mostly local, as autocrine/paracrine factors (Madeddu, Emanueli et al. 2007).

The angiotensin-converting enzyme (ACE), which is a key enzyme of the renin-angiotensin system, contributes to metabolic degradation of kinins. The ACE inhibitors (a class of anti-hypertensive drugs) have been successfully used to lower blood pressure in hypertensive patients. These drugs inhibit the enzyme ACE which normally converts angiotensin I to the biologically active peptide angiotensin II. In addition, ACE inhibitors were discovered to lower blood pressure by also allowing higher levels of kinins to circulate, since inhibition of ACE would result in decreased kinin degradation (Marceau and Regoli 2004; Madeddu, Emanueli et al. 2007).

Besides their cardioprotective effects, kinins contribute to pathophysiological states such as inflammatory diseases (for example, rheumatoid arthritis), bronchoconstriction

(asthma), myocardial infarction, and endotoxic shock (Regoli and Barabe 1980). It is clear that the KKS is an attractive therapeutic target because of its cardioprotective and nephroprotective effects, as well as its involvement in inflammatory processes.

BK is a nonapeptide with the amino acid sequence Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9. Lys-BK has the same sequence with an additional residue, an N-terminal lysine residue (Regoli and Barabe 1980). The KKS includes two kinin receptors that are GPCRs; the bradykinin types 1 and 2 receptors (B1R and B2R). B1R expression is induced by stresses such as inflammation, while B2R is constitutively expressed. BK and lys-BK bind with high affinity to B2R. On the other hand, des-Arg^9-BK and lys-des-Arg^9-BK (metabolic products of arginine carboxypeptidases that remove the C-terminal Arg residue in BK and lys-BK) bind B1R. The B1R, unlike B2R, does not readily desensitize upon activation and this may be mostly due to lack of phosphorylation and internalization of this receptor (Marceau and Regoli 2004).

The B1R and B2R are each coupled to and signal through the G protein G_q; hence, their activation results in phosphoinositol hydrolysis and an increase in intracellular Ca^{2+}. These receptors have also been found to couple to the G protein G_i (Madeddu, Emanueli et al. 2007). In endothelial cells, BK activates endothelial nitric oxide synthase (eNOS) in a calcium-dependent mechanism, which produces nitric oxide (NO), and NO in turn stimulates soluble guanylyl cyclase to produce cyclic guanosine monophosphate (cGMP). The production of cGMP results in vasodilation of vascular smooth muscle cells . BK also stimulates PKC-mediated activation of MAPK, contributing in turn to the activation of the calcium-sensitive, cytosolic phospholipases A_2. This results in prostacyclin (prostaglandin I_2) production, which in turn stimulates cAMP production. Production of

cAMP is another vasodilatory factor in vascular smooth muscle cells (Dimitropoulou, Chatterjee et al. 2006; Madeddu, Emanueli et al. 2007).

It appears from animal studies that both bradykinin receptors contribute to lowering blood pressure, but that a loss of function or inhibition of one receptor can be compensated for by the other (Duka, Duka et al. 2006). In addition to interacting with one another, the bradykinin receptors have also been shown to form heterodimers with angiotensin II receptors. B2R heterodimerization with the angiotensin II type 2 receptor (AT2R, also known to activate NO production) resulted in enhanced NO release (Abadir, Periasamy et al. 2006), while B2R and angiotensin II type 1 receptor (AT1R) heterodimerization showed enhanced Ang II action (AbdAlla, Abdel-Baset et al. 2005).

As previously mentioned, B2R activates Raf/MEK/MAPK via the $G_{q/11}$/PLC-β/PKC pathway. An alternative pathway of MAPK activation, via transactivation of the receptor tyrosine kinase epidermal growth factor receptor (EGFR), has been shown in COS-7 cells (in which B2R was artificially expressed) (Adomeit, Graness et al. 1999). The precise mechanism of how EGFR transactivation occurs is less understood, and it may involve either stimulation of a metalloproteinase or activation of a non-membrane tyrosine kinase (apparently a tyrosine kinase other than Src, since Src inhibition did not affect EGFR transactivation (Adomeit, Graness et al. 1999)). EGFR activation leads to activation of Ras and, subsequently, activation of the Raf/MEK/MAPK cascade. MAPK activation by endogenous B2 receptors has been demonstrated in a number of cells including endothelial cells, fibroblasts, rat ventricular myocytes, PC-12 cells, vascular smooth muscle cells and rat mesangial cells (Liebmann 2001).

Because of the mitogenic effects of the MAPKs, the role of BK-stimulated activation of these kinases is being studied in tumor cell lines. There have been interesting results regarding BK-mediated activation of MAPK in tumor cells. It appears that an alternative pathway to PKC-mediated MAPK activation in some tumor cell lines is via PI3K-associated EGFR without EGFR transactivation. In certain human colon (Graness, Adomeit et al. 1998), breast (Liebmann 2001) and endometrium (Liebmann 2001) carcinoma lines, BK did not induce EGFR transactivation. Intriguingly, BK stimulation of another human epidermal carcinoma cell line displayed EGFR trans-*inactivation* (Graness, Hanke et al. 2000); that is, a tyrosine-phosphatase-mediated decrease in both basal and EGF-stimulated tyrosine phosphorylation of EGFR (Liebmann 2001). The B2 receptor is thus a good model for elucidating new pathways of MAPK activation in tumor cell lines, and would help identify potential therapeutic targets.

1.8. Project rationale and objectives

The C-terminal tail of the B2R has been shown to contain important determinants for B2R endocytosis following BK stimulation. One study has implicated a cluster of serine/threonine residues in the tail region to be necessary for internalization of B2R (Pizard, Blaukat et al. 1999). This study employed mutants with truncations of and amino acid substitutions in the C-terminal tail. The mutants were expressed in three cell lines (COS, CHO and HEK 293) and their ligand binding, signaling, phosphorylation and internalization properties were compared. The C-terminal tail was found to be unimportant for BK ligand binding and activation of PLC and PLA_2. However, most of the mutants displayed reductions in internalization, and this effect was consistent between the different cell lines used. Deletion analyses hinted at a cluster of three serine and two threonine residues in a small area from positions 335 to 351, and point mutations to alanine were performed. When all five serine/threonine residues were mutated to alanine, the loss in internalization was comparable to deleting the entire segment from positions 335 to 351.

To better understand the mechanisms of ERK1/2 activation by the bradykinin type 2 receptor, we employed in our study different C-terminal mutants of the B2R, including mutations in the afore-mentioned serine/threonine cluster. One study suggested that mutating this cluster does not prevent the resulting internalization-defective receptor from activating ERK; however, this study did not investigate the temporal regulation of ERK activation (Blaukat, Pizard et al. 1999).

We employed a chimeric mutant of B2R in which its C-terminal tail after Cys-324 was replaced with that of the vasopressin V2 receptor. Our laboratory previously reported this receptor mutant's internalization and β-arrestin binding properties (Simaan, Bedard-

Goulet et al. 2005), but not its effects on ERK1/2 activation. This receptor mutant was named B2RV2CT. Another approach was generating mutants with amino acid substitutions. One of those, B2RV2CT6A, was produced from B2RV2CT by mutating all six residues of a serine/threonine cluster in the V2CT portion of the chimeric receptor. Three serine residues in this cluster have been previously reported to be important for preventing the V2 receptor from recycling (Innamorati, Sadeghi et al. 1998). A second receptor mutant generated by this approach was B2R5A, produced by amino acid substitutions in the serine/threonine cluster of wild-type B2R's C-terminal tail.

Even though internalization itself may not be an absolute requirement for MAPK activation by B2R (Blaukat, Pizard et al. 1999), this does not eliminate the possibility that β-arrestin may be involved in scaffolding the components of the MAPK cascade following B2R stimulation. Since this β-arrestin-dependent phase may exist, which has been shown for other receptors as previously mentioned but not for B2R, we expected that disruptions of potential β-arrestin binding sites in the B2R may lead to a change in the temporal kinetics of ERK1/2 activation.

2. Materials and methods

Materials

BK was purchased from Sigma Chemical Co. Transferrin conjugated to Alexa Fluor 568 was from Molecular Probes. Mouse monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) and rabbit anti-p44/42 MAP kinase antibodies were purchased from Cell Signaling Technology.

Plasmids and constructs

The human B2R (in pcDNA3) was a kind gift from Dr. R.C. Venema (Medical College of Georgia, GA). The B2RV2CT chimera, B2R-YFP and β-arrestin$_2$-CFP have been previously described (Simaan, Bedard-Goulet et al. 2005). B2RV2CT-YFP was generated by polymerase chain reaction (PCR). Briefly, the B2RV2CT chimera was amplified by PCR using a forward primer generating a *ClaI* site and a reverse primer removing the stop codon and generating a *SalI* site. The PCR product was cut *ClaI/SalI* and inserted into B2R-YFP cut with the same restriction enzymes. PCR was also used to generate β-arrestin$_2$T381-CFP. Briefly, a forward primer was that ended with the codon for amino acid 381 in β-arrestin$_2$ followed nonstop by a *KpnI* site and a reverse primer at an upstream region of β-arrestin$_2$ containing an *XhoI* site were used in order to amplify a region in β-arrestin$_2$-CFP. The β-arrestin$_2$T381-CFP was then obtained via three-way ligation of pECFP-N1 vector (Clontech) cut with *KpnI/HindIII*, the PCR fragment cut with *KpnI/XhoI*, and β-arrestin$_2$-CFP cut with *HindIII/XhoI*. B2RV2CT6A was generated by PCR, briefly as follows: a forward primer overlapping a *ClaI* site and a reverse primer, containing alanine mutations in the C-terminal serine/threonine clusters of the V2

receptor (Ser-362, Ser-363, Ser-364 and Thr-369, Ser-370, Ser-371), overlapping an *XbaI* site, were used to amplify a region in B2RV2CT. The PCR fragment was cut *ClaI/XbaI*. Wild-type human B2R was then cut with *ClaI* and *XbaI*, and ligated with the PCR fragment. B2R5A was constructed as follows: a forward primer overlapping a *ClaI* site and a reverse primer overlapping an *XhoI* site was used. The reverse primer contained alanine mutations as substitutions for Thr-342, Thr-345, Ser-346 and Ser-348. Full-length human B2R was used as template. The generated PCR fragment was cut *ClaI/XhoI*, and ligated with pcDNA3-B2R cut with *ClaI* and *XhoI*. It should be mentioned that a leucine to alanine mutation was erroneously introduced at positition 343, but was deemed to unlikely to cause any significant effects. Because the resulting mutant received 5 alanine substitutions, it was named B2R5A. B2R5A-YFP was generated by PCR as follows: a forward primer overlapping a *ClaI* site and a reverse primer overlapping a *SalI* site with killed stop were used, with B2R5A as template. The fragment was digested *ClaI/SalI*, and a three-way ligation was done with B2R-YFP (cut *ClaI/HindIII*) and pEYFP-N1 vector (Clontech; cut *HindIII/SalI*). B2RV2CT6A-YFP was generated with PCR, as follows: a forward primer overlapping a *ClaI* site and a reverse primer overlapping a *SalI* site with the stop killed were used, with B2RV2CT6A as template. The PCR fragment was cut *ClaI/SalI*, and a three-way ligation was done with B2R-YFP (cut *ClaI/HindIII*) and pEYFP-N1 vector (cut *HindIII/SalI*). All constructs were confirmed by sequence analysis at the Service d'Analyse et de Synthèse d'Acides Nucléiques, Université Laval, Québec, Canada.

Figure 9 illustrates the C-terminal portions of the wild-type B2R and the three mutant receptors, indicating the modifications made to the B2R in order to obtain the mutants.

Figure 9: The C-termini of wild-type B2 receptor and the mutant receptors generated. *A*, wild-type bradykinin type 2 receptor from residues 321 to 364, with ellipses representing upstream amino acids. This receptor contains 364 amino acids and is a shorter, alternatively spliced form. *B*, B2R5A mutant. The underlined residues were point-mutated to alanine. *C*, B2RV2CT chimeric mutant. Amino acids in bold, up to and including C324, are from the wild-type B2R. Amino acids in italics (A343 to S371) are the last 29 amino acids of the V2 receptor. *D*, B2RV2CT6A chimeric mutant, generated from B2RV2CT. Underlined residues represent point mutations to alanine.

A) Wild-type B2R

...Q321-G322-V323-C324-Q325-K326-G327-G328-C329-R330-S331-E332-P333-I334-Q335-M336-E337-N338-S339-M340-G341-T342-L343-R344-T345- S346-I347-S348-V349-E350-R351-Q352-I353-H354-K355-L356-Q357-D358-W359-A360-G361-S362-R363-Q364

B) B2R5A

...Q321-G322-V323-C324-Q325-K326-G327-G328-C329-R330-S331-E332-P333-I334-Q335-M336-E337-N338-S339-M340-G341-A342-A343-R344-A345-A346-I347-A348-V349-E350-R351-Q352-I353-H354-K355-L356-Q357-D358-W359-A360-G361-S362-R363-Q364

C) B2RV2CT

...**Q321-G322-V323-C324**-*A343-R344-G345-R346-T347-P348-P349-S350-L351-G352-P353-Q354-D355-E356-S357-C358-T359-T360-A361-S362-S363-S364-L365-A366-K367-D368-T369-S370-S371*

D) B2RV2CT6A

...**Q321-G322-V323-C324**-*A343-R344-G345-R346-T347-P348-P349-S350-L351-G352-P353-Q354-D355-E356-S357-C358-T359-T360-A361-A362-A363-A364-L365-A366-K367-D368-A369-A370-A371*

Cell culture and transfection

Human embryonic kidney (HEK293) cells were cultured in Minimal Essential Medium (MEM; VWR) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gibco), L-glutamine (2 mM; Invitrogen) and gentamicin (100 μg/ml; Invitrogen). HEK293 cells were transiently transfected in 6-well plates (at a density of 1.75×10^5 cells/well), in 35-mm glass bottom dishes (at a density of 1.2×10^5 cells/dish) and 100-mm culture dishes (at a density of 1.2×10^6 cells/dish) using the calcium phosphate co-precipitation method, in which plasmid DNA was mixed in a solution of 125 mM $CaCl_2$ buffered with 25 mM HEPES (pH 7.1), 140 mM NaCl and 0.75 mM Na_2HPO_4. Mouse embryonic fibroblast (MEF) wild-type and β-arrestin 1/β-arrestin 2 double-null cell lines were a kind gift from Dr. Robert J. Lefkowitz (Duke University, Durham, NC). The wild-type cell line is henceforth designated as MEF β-arr +/+, and the β-arrestin double-knockout cell line as MEF β-arr -/-. MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Gibco), L-glutamine (2mM; Invitrogen) and gentamicin (100 μg/ml; Invitrogen). MEF β-arr +/+ and MEF β-arr -/- cells were seeded in 6-well plates (at a density of 2.0×10^5 cells/well).

Bradykinin stimulation of cells and Western blotting

HEK293 cells were seeded in 6-well plates and transfected the second day with 0.5 μg/well of B2R, B2R5A, B2RV2CT or B2RV2CT6A. Fresh media was supplemented on the third day, and the experiments performed on the fourth day. The cells were first washed with warm MEM containing 20mM HEPES (for CO_2 buffering of the media to maintain a physiological pH level), and incubated in MEM-HEPES for 1 hour at 37 °C.

For stimulation of endogenous B2 receptors in MEF cells, cells were seeded in 6-well plates, serum-starved the second day overnight, and the experiments were done on the third day. The MEF cells were washed with and incubated in warm DMEM-HEPES for 1 hour at 37 °C. For both experiments, BK stimulation (final concentration of 10^{-6} M) was done at 37 °C in a water bath, starting with the longest time point and counting down to 0 min. Stimulation was stopped on ice with cold phosphate-buffered saline (PBS). The PBS was removed and the cells were solubilized with cold glycerol buffer (50 mM HEPES, 50 mM NaCl, 10% (vol/vol) glycerol, 0.5% (vol/vol) Nonidet P-40, and 2 mM EDTA) containing 100 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 2.5 μg/ml aprotinin, and 1 μg/ml Pepstatin A. Samples were rocked for 30 minutes at 4 °C, and centrifuged for 30 minutes at 4 °C (21,000 x *g*). 100 μl of each sample's supernatant was denatured in 100 μl of 2X Laemmli buffer (250 mM Tris-HCl pH 6.8, 2% SDS (wt/vol), 10% glycerol (vol/vol), 0.01% bromophenol blue (wt/vol) and 5% β-mercaptoethanol (vol/vol)) at 65 °C for 10 minutes. Proteins were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 10% milk in PBS/0.05% Tween-20 (vol/vol), and incubated with primary antibody overnight at 4 °C. The antibodies were used at the following dilutions: anti-phospho-p44/42 MAP kinase (1:2000 in 1% milk) and anti-p44/42 MAP kinase (1:2000 in 5% bovine serum albumin). The following day, membranes were washed with PBS-Tween and incubated in anti-mouse or anti-rabbit horse radish peroxidase antibody (1:10,000 dilution in 5% milk) for 1 hour. The membranes were washed again and immunoreactivity was visualized by enhanced chemiluminescence (SuperSignal West Pico, Pierce).

Live cell imaging

For co-localization experiments, HEK293 cells seeded in 35 mm glass-bottom dishes were transfected the second day with the following amounts of plasmid: 0.5 μg/dish of B2R-YFP, B2R5A-YFP, B2RV2CT-YFP or B2RV2CT6A-YFP, along with 0.083 μg/dish of β-arrestin$_2$-CFP or β-arrestin$_2$T381-CFP. The cells were given fresh media on the third day. The experiments were performed on the fourth day of culture. First, the plates were washed with and incubated in MEM-HEPES for 30 minutes at 37 °C. The fluorescence of the YFP-tagged receptors and CFP-tagged β-arrestins was visualized with a Zeiss LSM-510 META laser scanning microscope with a 60x oil immersion lens. Multitrack mode was used with dual excitation (458 nm for CFP and 514 nm for YFP) and emission (BP 470-500 nm for CFP, and BP 530-600 nm for YFP) filter sets. The plates were placed in a heating stage set at 37 °C. A picture was taken for each 0-minute time point before stimulation with BK (10^{-6} M final concentration) was initiated. Subsequent pictures were taken at the indicated time points. For colocalization of B2R-YFP and B2R5A-YFP with transferrin, cells were transfected with 0.5 μg/dish of B2R-YFP or B2R5A-YFP, and cultured as above. On the day of the experiment, the dishes were washed with and incubated in warm MEM-HEPES for 30 minutes at 37 °C. The cells were simultaneously stimulated with BK (10^{-6} M final concentration) and transferrin conjugated with Alexa Fluor 568 (final concentration of 50 μg/ml) for 15 minutes at 37 °C, followed by two washes with warm media to remove unbound transferrin. Pictures were immediately taken using the Zeiss LSM-510 META laser scanning microscope with a 60x oil immersion lens, using dual excitation (514 nm for YFP and 543 nm for Alexa Fluor 568) and emission (BP 530-600 nm for YFP and LP 560 nm for Alexa Fluor 568)

filter sets. Final figures were collated using Adobe Photoshop 7.0 and Illustrator 10.0. Results are representative of at least 3 independent experiments.

FACS analysis

HEK293 cells were seeded in 100-mm dishes and transfected the second day with 3 μg/dish empty vector, 3 μg/dish B2R-YFP or 3 μg/dish 5A-YFP (with two dishes for each point). The cells were given fresh media on the third day. On the fourth day, the plates were carefully washed with room temperature PBS 1X, and gently scraped into clear tubes with PBS containing 2mM EDTA. The samples were analyzed by fluorescence-activated cell sorting at the Flow Cytometry Core of the Institut de Recherches Cliniques de Montréal. The FACS analysis was performed in 2 independent experiments with similar results.

Data analysis

Western blot band intensities were quantified by densitometry analysis using MetaMorph imaging software. Results are presented as means ± S.E.M. of at least 3 independent experiments.

3. Results

3.1. Modification of the C-terminus of the B2 receptor leads to differential temporal activation patterns of ERK1/2

We wished to see changes, if any, in the temporal pattern of ERK1/2 activation when the B2 receptor is mutated in its C-terminus. The wild-type B2 receptor and the mutant receptors B2R5A, B2RV2CT and B2RV2CT6A were transfected in HEK 293 cells. *Figure 10* shows the time course of ERK1/2 activation by the wild-type B2R and the three mutant receptors. Stimulation with BK was done for the times indicated in the figure.

Wild-type B2R maximally activated ERK1/2 at 5 minutes. Activation of ERK1/2 then decreased to lower levels from 15 to 60 minutes, eventually reaching phospho-ERK1/2 intensity of about 6% at 60 minutes, compared to the maximal intensity observed at 5 minutes.

In marked contrast, the B2R5A mutant-mediated ERK1/2 activation had a significantly higher intensity in the short-term phase, with a maximum peak at 140% relative to wild-type B2R. The decrease in ERK1/2 activation was very gradual, and even at 60 minutes it remained sustained at about 50%.

The B2RV2CT chimera activated ERK1/2 at 5 minutes of stimulation at a level about 10% greater than the wild-type receptor. The intensity of activation dropped gradually, but it still remained higher than that of the wild-type B2R by 60 minutes of BK stimulation (with levels at about 20%, compared to 6% for the wild-type receptor).

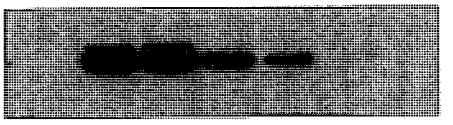
The B2RV2CT6A mutant initially activated ERK1/2 with similar intensities as the B2RV2CT chimera, but the ERK1/2 phosphorylation induced by its activation eventually reached basal values (around 1% relative to maximal wild-type receptor activation).

Figure 10: Temporal activation of ERK1/2 by wild-type B2 receptor as compared to mutants of the B2 receptor's C-terminus. *A*, immunoblots showing BK-stimulated phosphorylation of ERK1/2 by B2R, B2R5A, B2RV2CT and B2RV2CT6A for the indicated time points. Total ERK1/2 for each time point is shown as a loading control. *B*, quantified phospho-ERK1/2 intensities, with values as percentages of the intensity at 5 minutes for wild-type B2R (set to 100). Results are presented as means ± S.E.M. of 4 independent experiments.

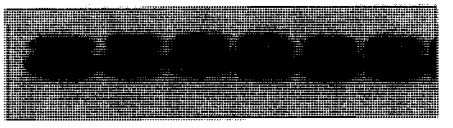
A

Wild-type B2R

0 2 5 15 30 60



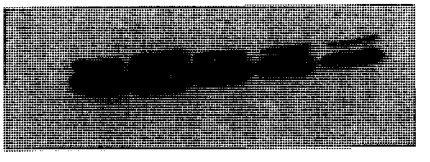
Phospho-ERK1/2



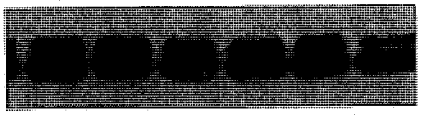
Total ERK1/2

B2R5A

0 2 5 15 30 60



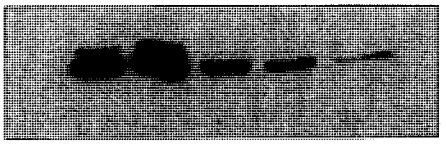
Phospho-ERK1/2



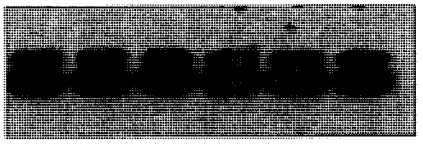
Total ERK1/2

B2RV2CT

0 2 5 15 30 60



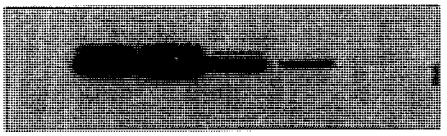
Phospho-ERK1/2



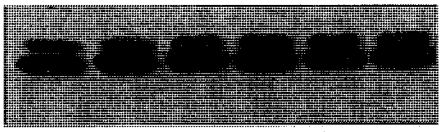
Total ERK1/2

B2RV2CT6A

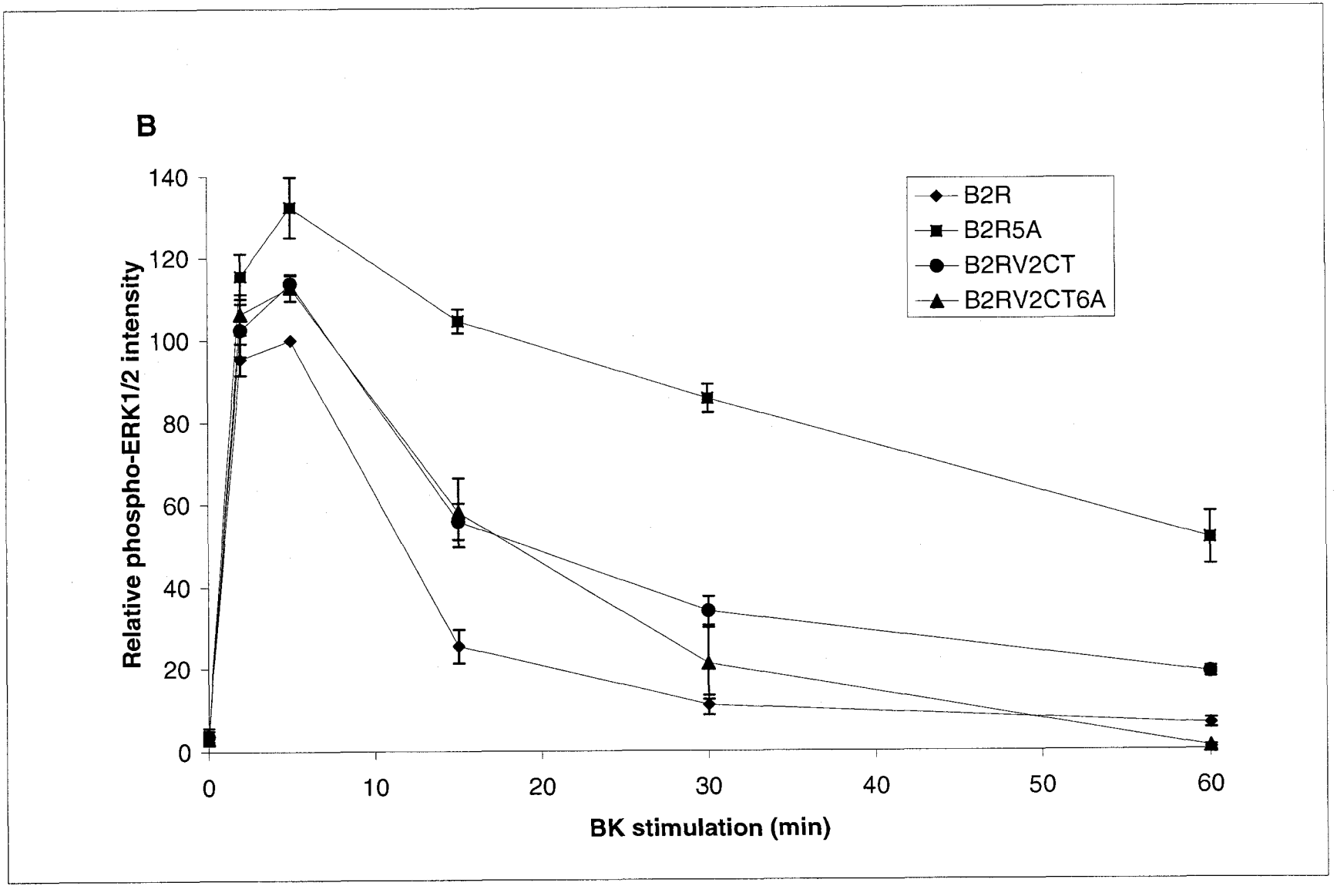
0 2 5 15 30 60



Phospho-ERK1/2



Total ERK1/2


B
Relative phospho-ERK1/2 intensity
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
BK stimulation (min)
B2R
B2R5A
B2RV2CT
B2RV2CT6A

3.2. Mutants of the B2 receptor C-terminus display differential β-arrestin-binding and internalization patterns

Since the sites of mutation in the B2 receptor may be important for receptor binding to β-arrestin, we investigated the colocalization of these mutants with β-arrestin$_2$ using confocal microscopy. The wild-type and mutant receptors were YFP-tagged and β-arrestin$_2$ was CFP-tagged. Pictures of unstimulated cells and cells at 2 and 15 minutes of BK stimulation are shown in *figure 11*.

Our laboratory previously reported that wild-type B2R colocalizes with β-arrestin$_2$ into endosomes (Simaan, Bedard-Goulet et al. 2005), as seen in *figure 11A*. At 0 minutes of stimulation, the wild-type B2 receptor was localized at the plasma membrane, and β-arrestin$_2$ was distributed evenly in the cytosol. At 2 minutes of stimulation, β-arrestin$_2$ appeared to translocate to the plasma membrane, clustering in pits. By 15 minutes of stimulation, B2R and β-arrestin$_2$ colocalized in endosomes as expected.

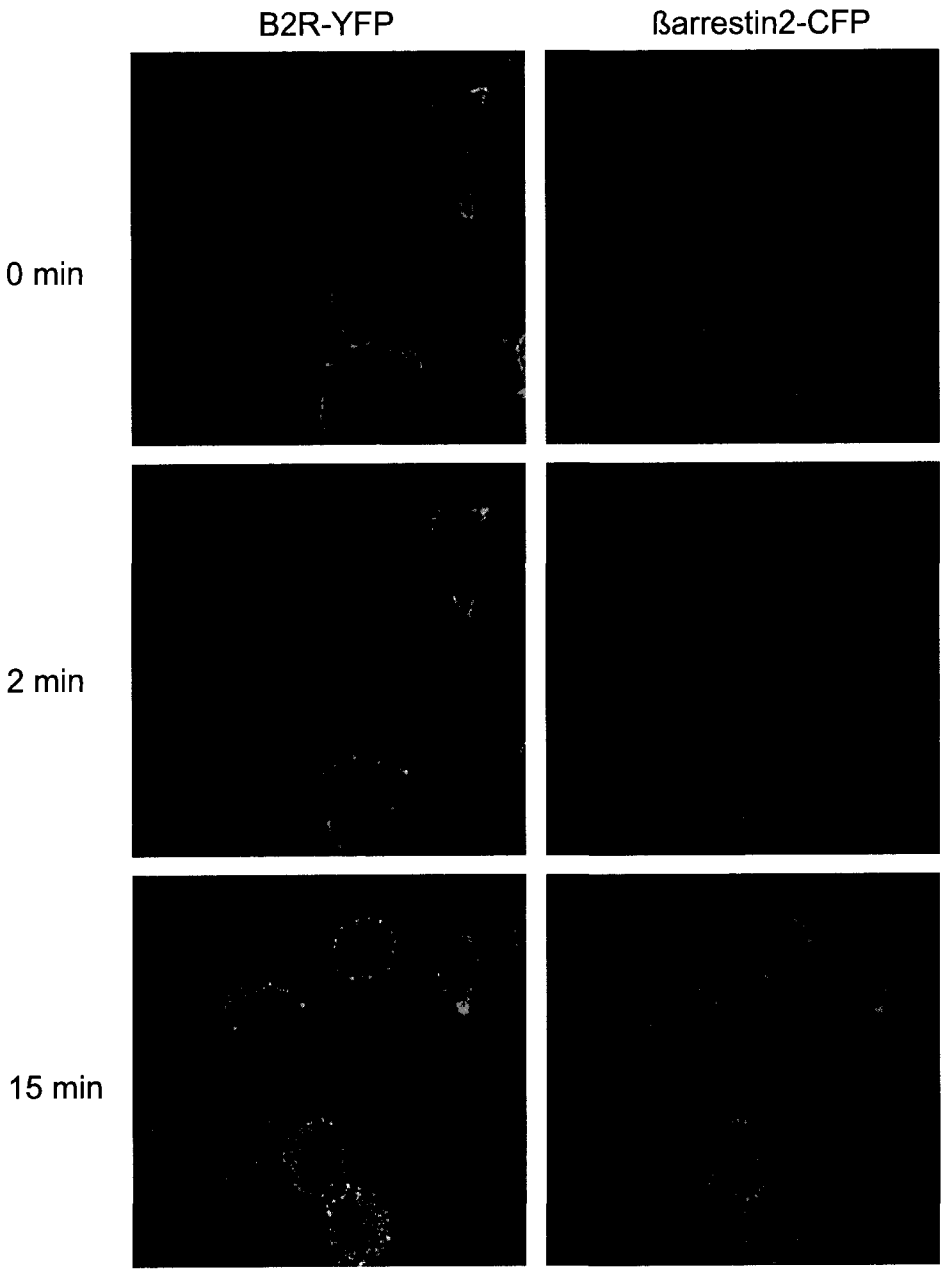
Since mutations of the serine/threonine cluster in B2R have been shown to greatly reduce internalization of the receptor (Pizard, Blaukat et al. 1999), we suspected that this may be due to the inability of the receptor to bind β-arrestin, since putative target sites of receptor-desensitizing kinases such as GRK were lost. *Figure 11B* shows cytosolic distribution of β-arrestin$_2$ at 0 minutes of BK stimulation, with the receptor mutant B2R5A at the cell surface. Stimulation with BK for 2 minutes did not lead to β-arrestin$_2$ translocation to the plasma membrane. Further stimulation (15 minutes) resulted in blunted internalization of B2R5A, consistent with previous studies that used radioligand binding to monitor mutant B2R internalization (Pizard, Blaukat et al. 1999). There was no colocalization with β-arrestin$_2$ in the endosomes that did eventually form.

Shown next in *figure 11C* is the B2RV2CT chimera, which our laboratory has previously reported to bind β-arrestin$_2$ with more avidity than wild-type B2 receptor (Simaan, Bedard-Goulet et al. 2005). At 0 minutes of BK stimulation, the mutant was distributed at the cell surface and β-arrestin$_2$ was in the cytosol. Translocation of β-arrestin$_2$ to the plasma membrane was clearly seen at 2 minutes of stimulation, and by 15 minutes the B2RV2CT mutant and β-arrestin$_2$ colocalized in endosomes, as expected.

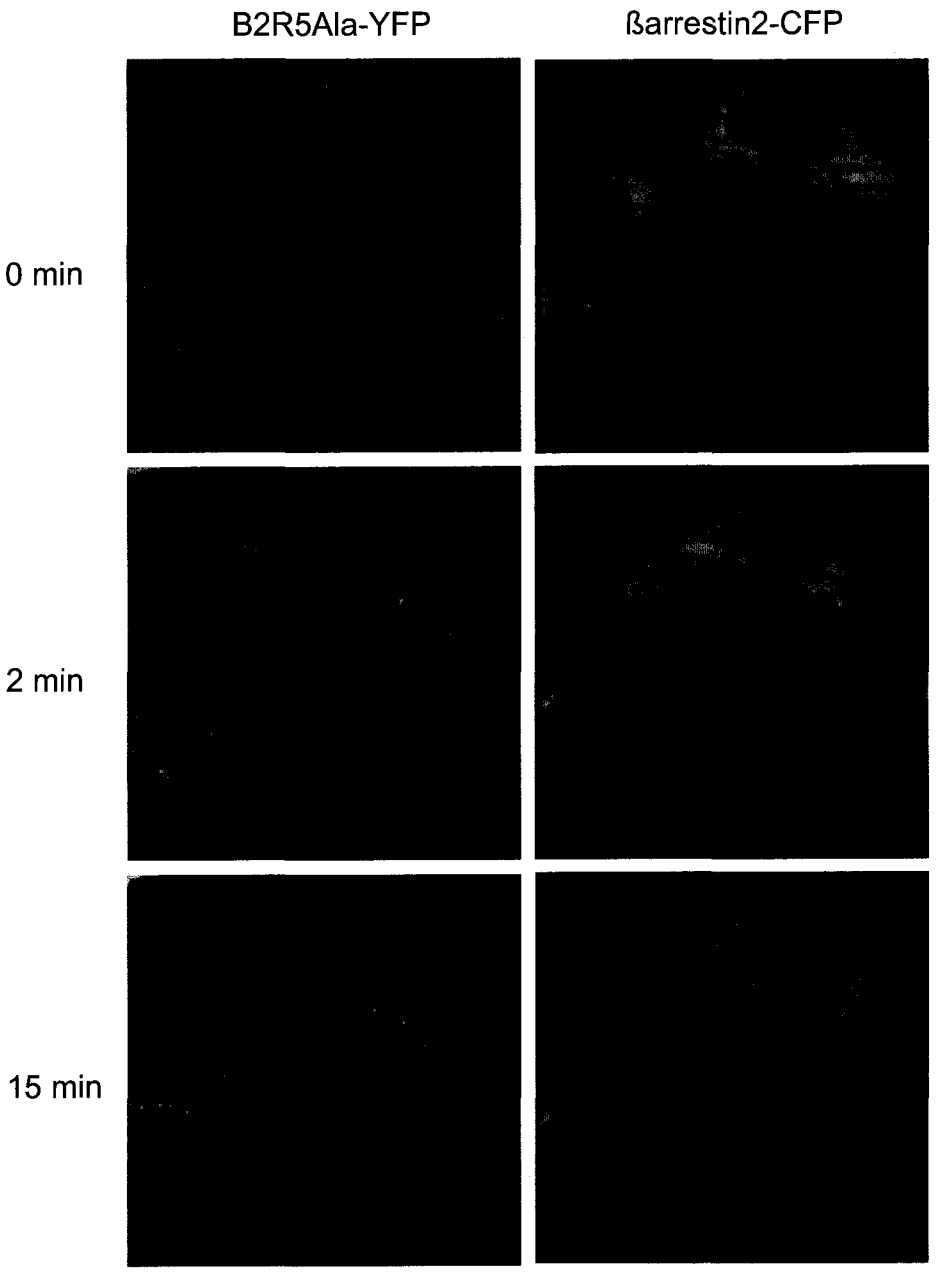
Finally, the same experiment was done on B2RV2CT6A, as seen in *figure 11D*. The mutant receptor was at the cell surface and β-arrestin$_2$ was in the cytosol before stimulation was initiated. At 2 minutes of stimulation, there was seemingly no difference with the basal state. By 15 minutes, however, the mutant receptor was seen in endosomes, but unlike with the wild-type B2R and the B2RV2CT chimera, there was no colocalization with β-arrestin$_2$.

Figure 11: Colocalization studies of YFP-tagged receptors with β-arrestin$_2$-CFP following BK stimulation at the indicated time points. *A*, wild-type B2R. *B*, B2R5A. *C*, B2RV2CT. *D*, B2RV2CT6A. These results are representative of at least 3 independent experiments.

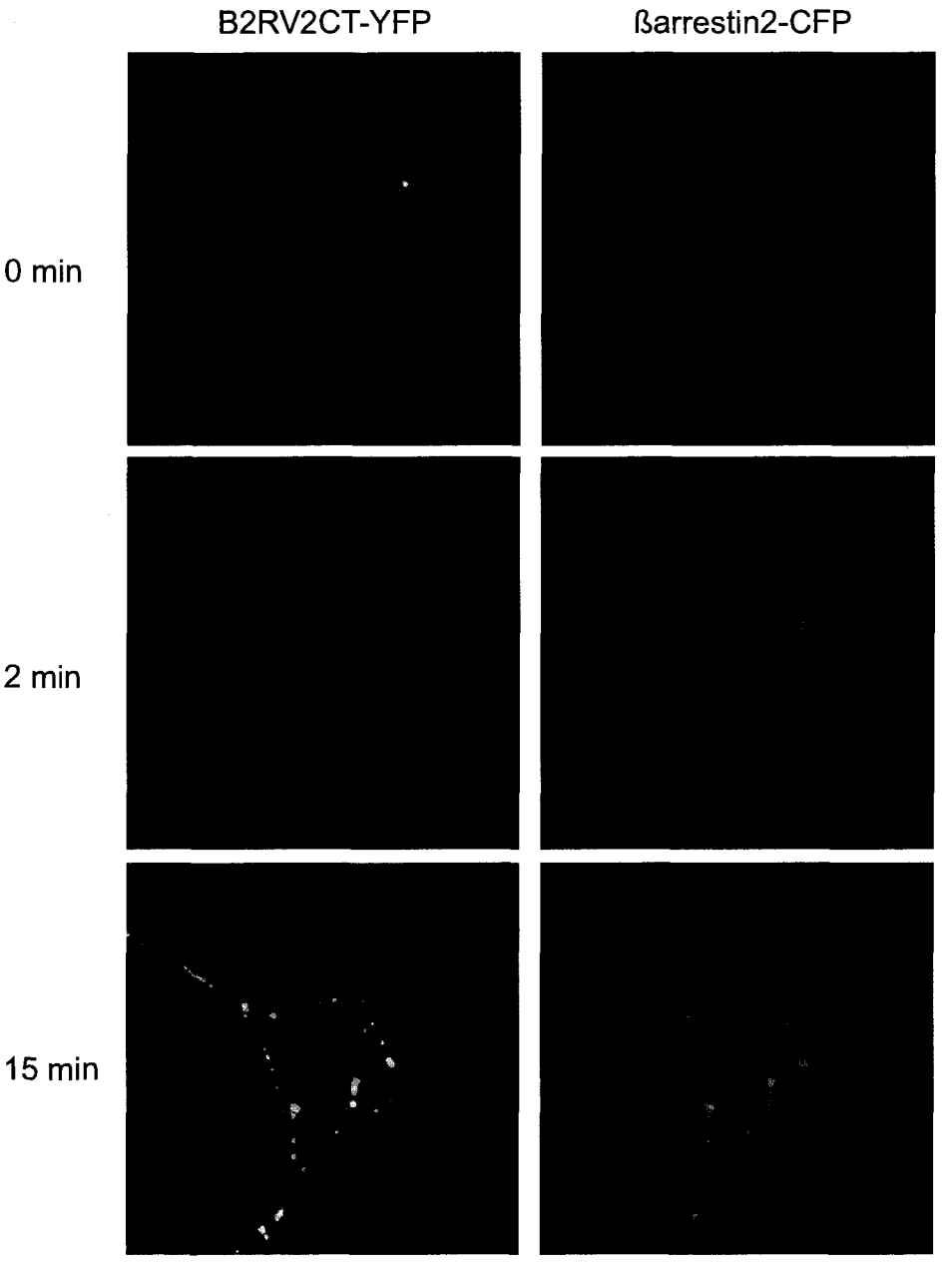
A



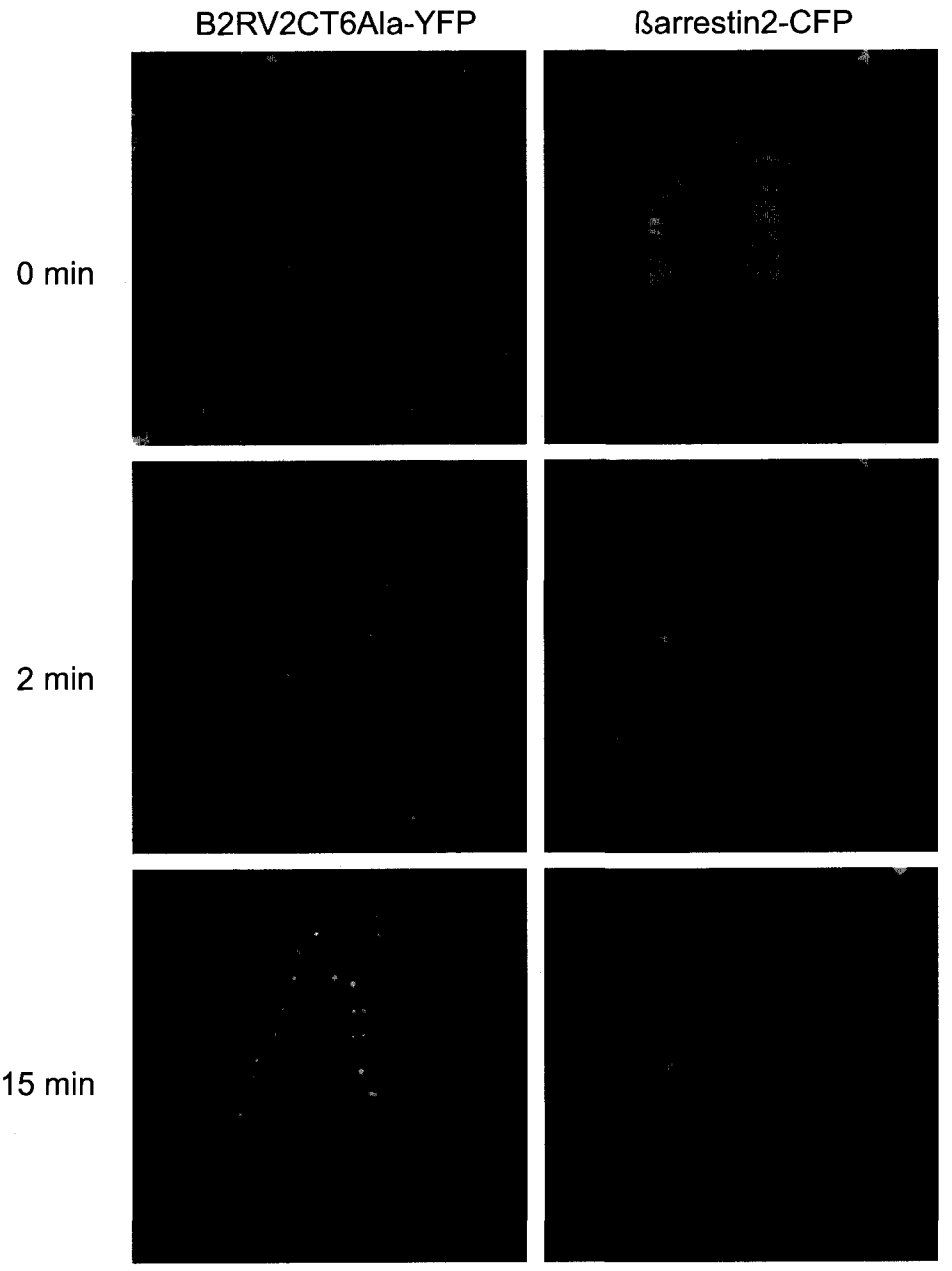
B



C



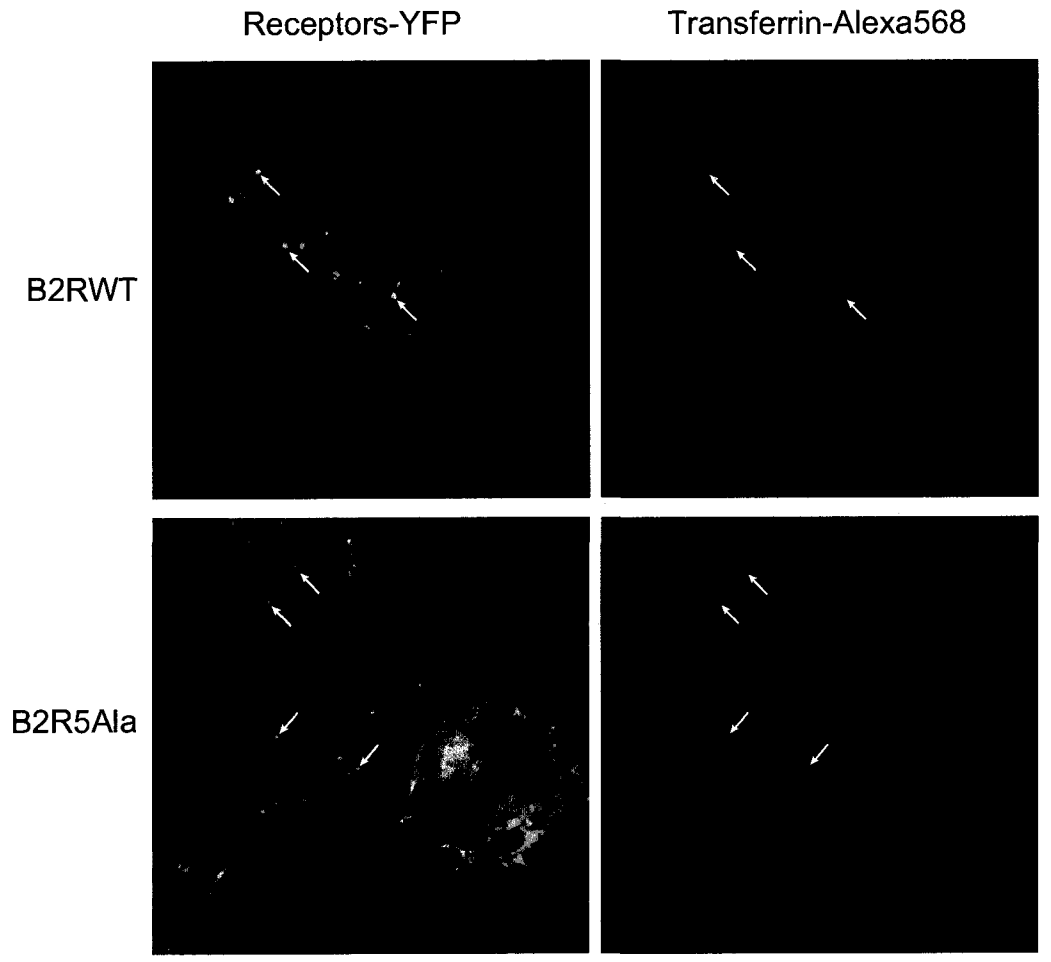
D



3.3. Characterization of the identity of the endosomes containing B2R5A mutant receptors as early endosomal vesicles

We wished to identify the nature of the endosomes that formed upon BK stimulation of the receptor mutant B2R5A.We used transferrin conjugated to Alexa Fluor 568 for this purpose. The transferrin receptor is a well-established marker for early endosomes (Hopkins 1983; Schmid, Fuchs et al. 1988). Cells transfected with either YFP-tagged wild-type B2R or YFP-tagged B2R5A were stimulated with BK and conjugated transferrin simultaneously for 15 minutes, and washed thoroughly before pictures were taken with the confocal microscope. Seen in the upper two panels of *figure 12* is wild-type B2R colocalization with transferrin into early endosomes (*white arrows*), as expected. The lower two panels, in which cells expressed B2R5A, depict that there is colocalization between transferrin and the mutant B2R5A (*white arrows*), confirming that the vesicles that form by 15 minutes of BK stimulation of B2R5A are indeed early endosomal vesicles.

Figure 12: Internalization of B2R or B2R5A simultaneously with transferrin in early endosomes. White arrows indicate endosomes that contain colocalized YFP-tagged receptor and Alexa Fluor 568-tagged transferrin. These results are representative of 3 independent experiments.


Receptors-YFP
Transferrin-Alexa568
B2RWT
B2R5Ala

3.4. β-arrestin$_2$ truncated at position 381 binds wild-type B2R but not B2R5A

Next, we attempted to force the B2R5A mutant into binding with β-arrestin in order to modulate the temporal ERK1/2 activation pattern of the mutant receptor. The rationale behind this approach was that forced β-arrestin-binding would presumably lead to increased internalization rates of the mutant receptor, thus dampening its highly sustained ERK1/2 activation. A β-arrestin$_2$ mutant truncated at position 381 (β-arrestin$_2$T381) was used. This mutant is missing the nuclear export signal in its C-terminus (L395/L397) that normally keeps the wild-type β-arrestin$_2$ cytoplasmic only (however, wild-type β-arrestin$_1$ lacks this signal and shows even cytoplasmic and nuclear distributions). Thus the β-arrestin$_2$T381 mutant localizes in both the nucleus and the cytoplasm (Scott, Le Rouzic et al. 2002). This mutant has been shown to bind agonist-occupied receptors with greater avidity than the wild-type β-arrestin$_2$ does (Kovoor, Celver et al. 1999; Oakley, Laporte et al. 2000), and is believed to be insensitive to the GRK-mediated phosphorylation state of the receptor. Thus, we rationalized that this truncated β-arrestin$_2$ mutant may be able to bind to the receptor mutant B2R5A, in contrast to wild-type β-arrestin$_2$ that did not exhibit translocation to or colocalization in endosomes with B2R5A (*figure 11B*). Work from our laboratory has previously shown that β-arrestin$_2$T381 forms a more stable complex than β-arrestin$_2$ with the wild-type B2 receptor, trapping the receptor and, subsequently, not allowing it to recycle and resensitize (Simaan, Bedard-Goulet et al. 2005).

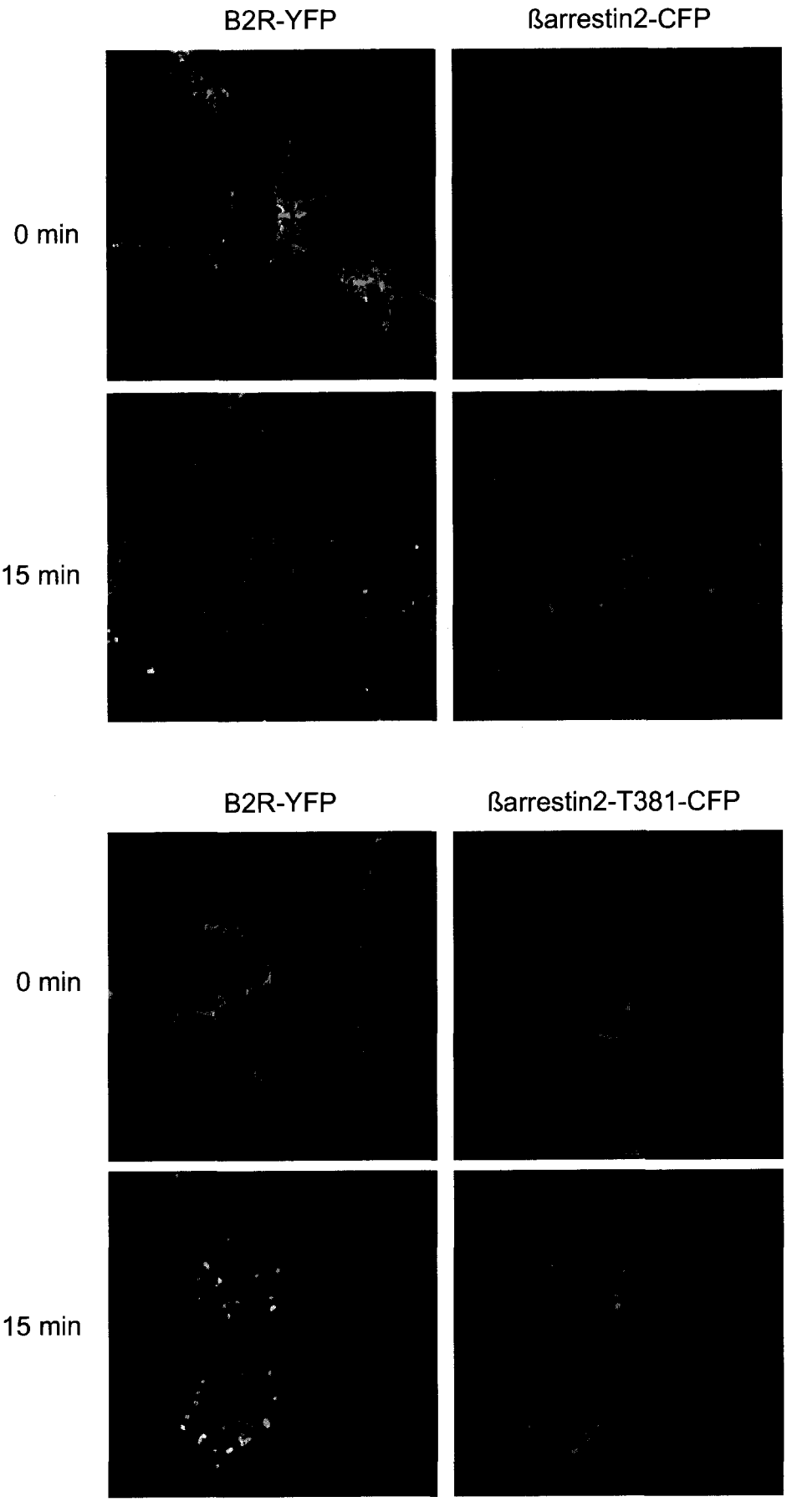
Figure 13 shows confocal microscopy images of HEK293 cells transfected with wild-type B2R or mutant B2R5A, along with β-arrestin$_2$ or β-arrestin$_2$T381. In *figure 13A*, cells were transfected with YFP-tagged wild-type B2R that was co-expressed with either CFP-tagged β-arrestin$_2$ or β-arrestin$_2$T381. At 0 minutes of BK stimulation, the receptor

was at the plasma membrane and both β-arrestins were cytosolic (with the β-arrestin$_2$T381 additionally seen in the nucleus, as previously mentioned) . At 15 minutes of stimulation, the B2 receptor colocalized well with each of the wild-type and mutant β-arrestin, as expected.

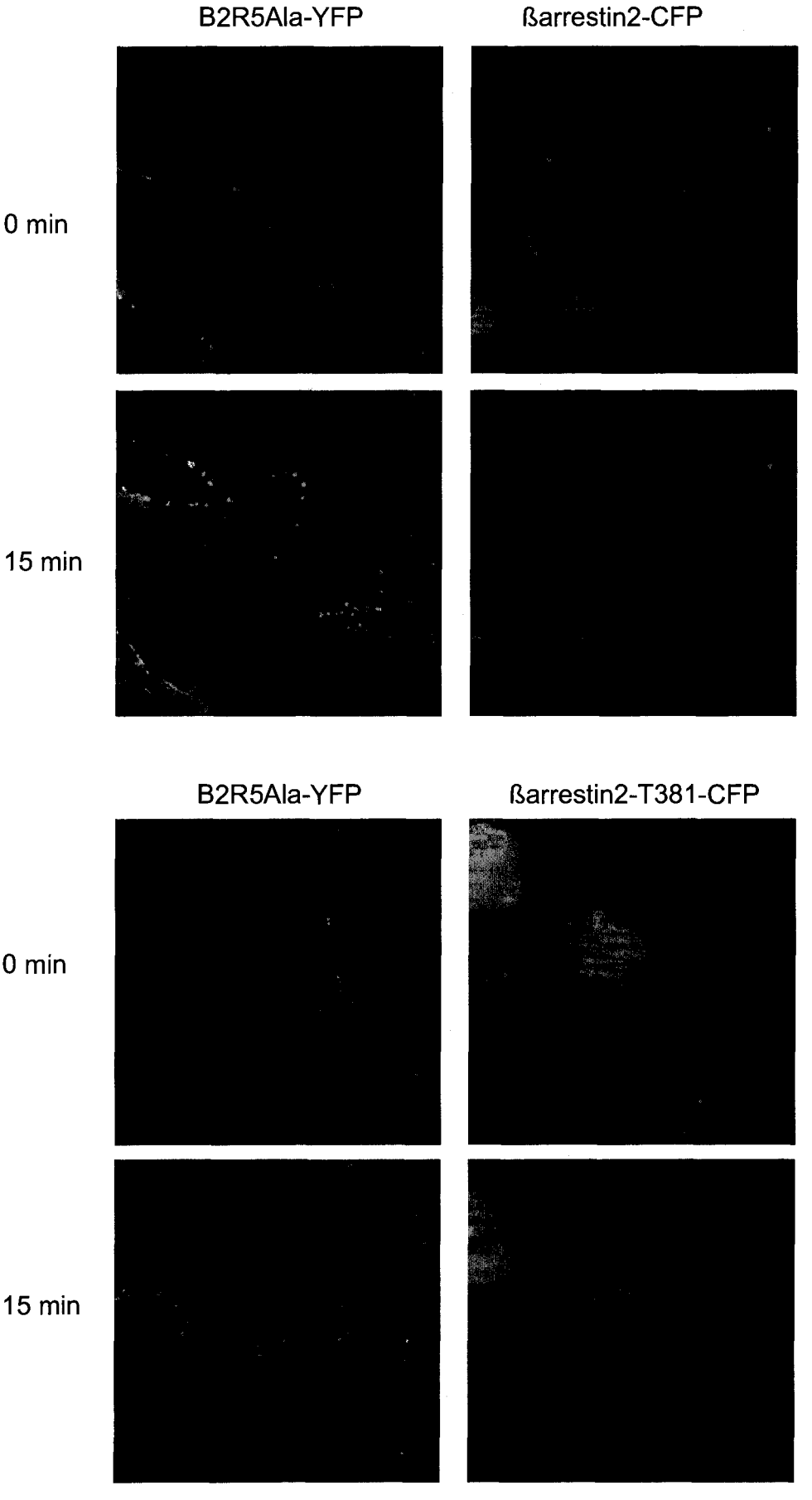
Figure *13B* shows cells transfected with the YFP-tagged mutant B2R5A, co-expressed with either CFP-tagged β-arrestin$_2$ or β-arrestin$_2$T381. Before stimulation was initiated, the receptor was at the plasma membrane. β-arrestin was cytosolic (wild-type β-arrestin$_2$) or both cytosolic and nuclear (β-arrestin$_2$T381). In the case of B2R5A-YFP co-expressed with wild-type β-arrestin$_2$-CFP, results obtained at 15 minutes of BK stimulation were similar to those in *figure 11B*; there was no colocalization, and internalization of the B2R5A mutant was less robust than that of wild-type receptor. When β-arrestin$_2$T381 was employed, similar results were obtained: there was some B2R5A internalization via endosomes, but no colocalization with β-arrestin$_2$T381. Thus, increased avidity of the truncated β-arrestin$_2$ to the mutant B2R5A could not be achieved.

Figure 13: Colocalization studies of YFP-tagged B2R or B2R5A with either β-arrestin$_2$-CFP or β-arrestin$_2$T381-CFP. BK stimulation was done for 0 or 15 minutes. *A*, wild-type B2R. *B*, B2R5A. These results are representative of 3 independent experiments.

A


B2R-YFP
ßarrestin2-CFP
0 min
15 min
B2R-YFP
ßarrestin2-T381-CFP
0 min
15 min

B

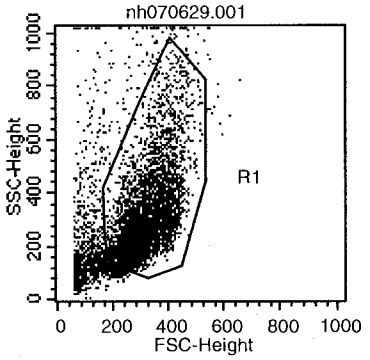

B2R5Ala-YFP
ßarrestin2-CFP
0 min
15 min
B2R5Ala-YFP
ßarrestin2-T381-CFP
0 min
15 min

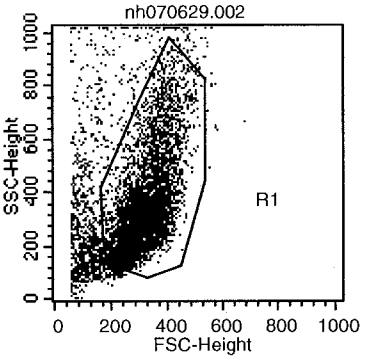
3.5. The sustained activation of ERK1/2 by the mutant B2R5A as compared to wild-type receptor is not due to differences in levels of receptor expression

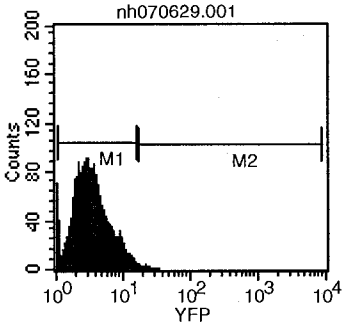
To exclude the possibility that the sustained ERK1/2 activation by the mutant B2R5A (*fig. 10*) was a result of differences in receptor expression, FACS analysis was performed on HEK 293 cells transfected with equal amounts of B2R-YFP, B2R5A-YFP or empty vector (as a negative control). The analysis results are shown in *figure 14*. The gate window M2 represents the populations of cells for each sample that are fluorescent. The negative control samples (*figure 14A*) had negligible fluorescence, as expected. The B2R-YFP- (*figure 14B*) and B2R5A-YFP-transfected cells (*figure 14C*) displayed very similar fluorescence intensities, and thus it was concluded that the B2R5A mutant was not expressed in significantly higher amounts than the wild-type B2R.

Figure 14: FACS analysis of cells transfected with equal amounts of empty vector (negative control), B2R-YFP or B2R5A-YFP (two samples per negative control or receptor). *A*, negative controls. *B*, B2R-YFP. *C*, B2R5A-YFP. SSC and FSC stand for side scatter and forward scatter, respectively, and are used to exclude dead cells and debris from analysis. The gate window M1 represents the population of cells with very little or no fluorescence. The gate window M2 represents the fluorescent population of each sample. Fluorescence intensity ("YFP") is represented on the x-axis, and cell counts on the y-axis. Results are representative of two independent experiments.

A







Histogram Statistics

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Log Data Units: Linear Value
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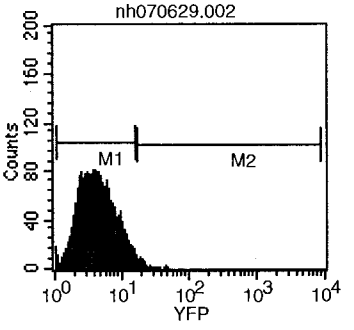
Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 10819
Total Events: 13258

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 991(	10819	100.00	81.60	3.87
M1	1, 1(	10668	98.60	80.46	3.82
M2	17, 866(	38	0.35	0.29	21.50



Histogram Statistics

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Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 11595
Total Events: 14178

Marker	Left, Right	Events	% Gated	% Total	Mean
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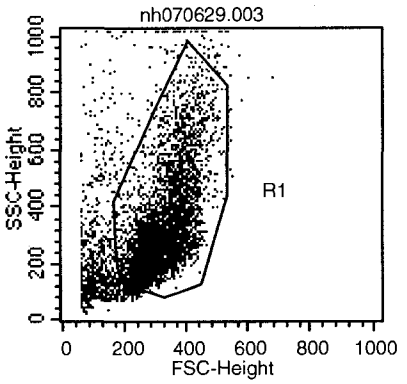
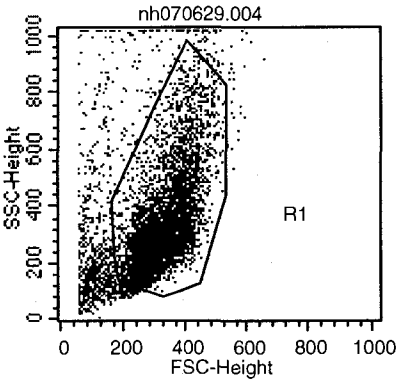
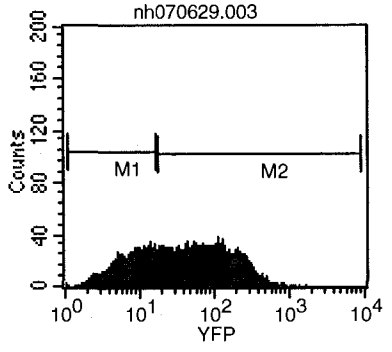
M1	1, 1(	11430	98.58	80.62	4.73
M2	17, 866(	102	0.88	0.72	21.48

B







Histogram Statistics

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Log Data Units: Linear Values
Sample ID: B2R 3ug #1
Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 10968
Total Events: 13239

Marker	Left, Right	Events	% Gated	% Total	Mean
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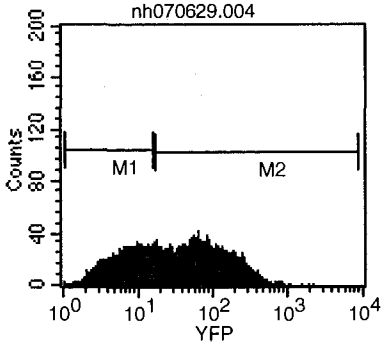
M2	17, 8660	7247	66.07	54.74	101.37

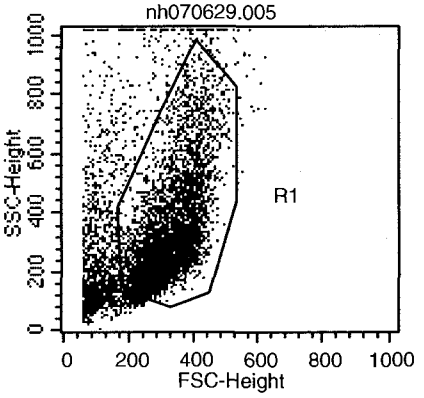


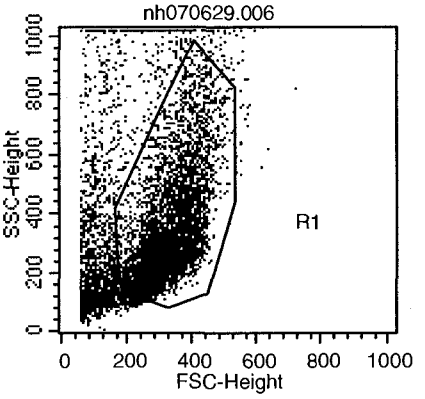
Histogram Statistics

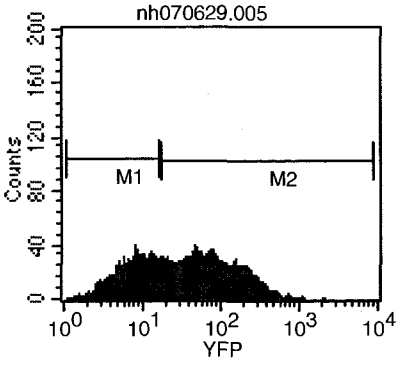
File: nh070629.004
Log Data Units: Linear Values
Sample ID: B2R 3ug #2
Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 11305
Total Events: 13403

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	11305	100.00	84.35	62.40
M1	1, 16	4161	36.81	31.05	7.65
M2	17, 8660	6971	61.66	52.01	96.23

C







Histogram Statistics

File: nh070629.005
Log Data Units: Linear Values
Sample ID: 5A 3ug #1
Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 11253
Total Events: 14728

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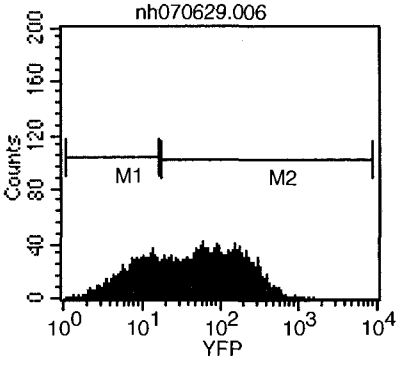
M2	17, 8660	6928	61.57	47.04	98.38



Histogram Statistics

File: nh070629.006
Log Data Units: Linear Values
Sample ID: 5A 3ug #2
Acquisition Date: 29-Jun-07
Gate: G1
Gated Events: 11481
Total Events: 15482

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 991C	11481	100.00	74.16	79.18
M1	1, 1E	3373	29.38	21.79	8.49
M2	17, 8660	7961	69.34	51.42	110.29

3.6. β-arrestin promotes B2R-mediated ERK activation

Past studies have demonstrated that β-arrestin can act as a scaffold for a growing list of proteins such as the members of the MAPK family, including ERK1/2 (Luttrell 2005). This has not yet been shown for the bradykinin type 2 receptor. To investigate the possibility that β-arrestin$_2$ can scaffold the components of the ERK1/2 cascade following activation of wild-type B2R, we employed a mouse embryonic fibroblast cell line double-null for both β-arrestin$_1$ and β-arrestin$_2$ (abbreviated as MEF β-arr -/-) and compared it to a wild-type cell line (MEF β-arr +/+). *Figure 15* shows results obtained when endogenous B2 receptors in these two MEF cell lines were stimulated with BK for the indicated times. At 2 minutes of stimulation, there were no significant differences. However, from 5 minutes of stimulation onwards there was a divergence in the fold over basal intensities, with the B2 receptors of the MEF β-arr -/- cell line activating ERK1/2 to a markedly lesser extent than endogenous receptors of the wild-type MEF cell line. The loss of both β-arrestins 1 and 2 appears to be accompanied by a decrease in the fold-over-basal intensity of activated ERK1/2, preliminary evidence that in the case of the B2 receptor, β-arrestin can scaffold components of the ERK1/2 pathway, presumably facilitating the interaction of the various kinases in the cascade with one another, thus promoting ERK1/2 activation (late phase ERK activation, as opposed to the short, initial phase of activation downstream of G proteins).

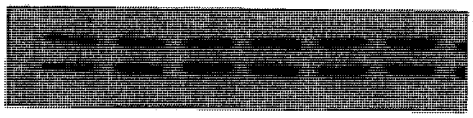
Figure 15: β-arrestin promotes B2R-mediated ERK activation. *A*, immunoblots showing BK-stimulated phosphorylation of ERK1/2 for the indicated time points by endogenous B2 receptors in MEF β-arr +/+ and MEF β-arr -/- cell lines. Total ERK1/2 for each time point is shown as a loading control. *B*, quantified phospho-ERK1/2 intensities, with values represented as fold over basal. Results are presented as means ± S.E.M. of 3 independent experiments.

A

MEF beta-arr +/+

0 2 5 10 15 30

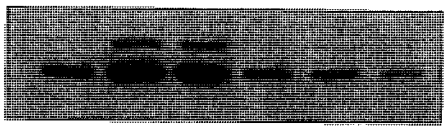
Phospho-ERK1/2



Total ERK1/2

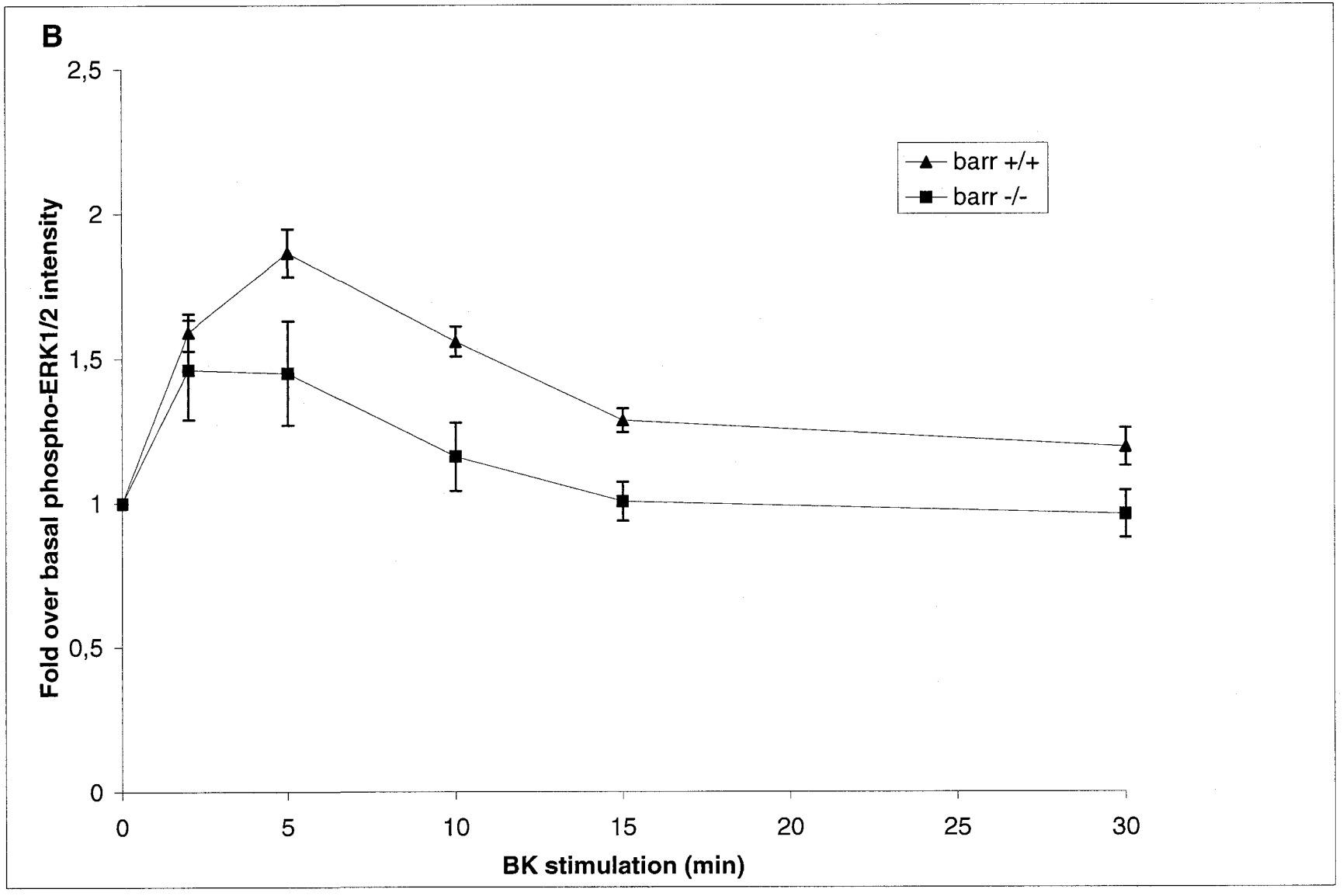
MEF beta-arr -/-

0 2 5 10 15 30



Phospho-ERK1/2

Total ERK1/2


B
2,5
2
1,5
1
0,5
0
Fold over basal phospho-ERK1/2 intensity
barr +/+
barr -/-
0
5
10
15
20
25
30
BK stimulation (min)

4. Discussion

The purpose of the present study was to better understand the ERK1/2 MAPK activation downstream of the bradykinin type 2 receptor (B2R). For this purpose, we employed mutants of the receptor's C-terminus, a region that is known to contain important determinants for desensitization and internalization (Pizard, Blaukat et al. 1999), a mutant β-arrestin construct that our laboratory has previously shown to bind wild-type B2R with greater avidity compared to wild-type β-arrestin (Simaan, Bedard-Goulet et al. 2005; Gousseva, Simaan et al. 2008), and a mouse embryonic fibroblast cell line double-null for β-arrestin 1 and 2.

4.1. Modulation of the C-terminus of the bradykinin type 2 receptor results in differential internalization rates, β-arrestin binding, and ERK1/2 temporal activation patterns

We found that mutating the clusters of serine/threonine residues in the B2 receptor's C-terminus (a mutant we named B2R5A) resulted in the mutant receptor displaying a marked decrease in internalization, as expected. In addition, this mutant was found to lose the β-arrestin binding that is normally displayed by the wild-type receptor.

The B2RV2CT6A mutant was a modification of a B2RV2CT chimera that was previously reported by our laboratory (Simaan, Bedard-Goulet et al. 2005). This mutant consisted of alanine mutations of two clusters of serine/threonine residues in the C-terminal region (the portion that originated from the terminal 29 amino acids of the vasopressin V2 receptor). A previous study has shown that one of these V2 receptor C-terminal clusters (Ser-362, Ser-363 and Ser-364) is important for preventing the receptor from recycling (Innamorati, Sadeghi et al. 1998), with mutation of any of these serine residues resulting in V2R gaining the ability to recycle back to the cell surface, as

opposed to being trapped intracellularly and eventually targeted for degradation. In the present study, the B2RV2CT6A mutant displayed transient β-arrestin binding but internalized robustly, unlike the comparable mutant B2R5A (whose C-terminal serine/threonine clusters were similarly mutated). These results suggest that the serine/threonine cluster in the C-terminal tail of wild-type bradykinin B2 receptor is important for both receptor internalization and β-arrestin binding, while in the case of the vasopressin V2 receptor the equivalent cluster is important for β-arrestin binding but is dispensable for internalization.

These differences may be inherent properties of GPCRs. In the case of the B2RV2CT chimera with mutated serine/threonine clusters (B2RV2CT6A), the mutation caused the chimera to exhibit properties of class A GPCRs (transient β-arrestin binding but robust internalization), as compared to the B2RV2CT chimera that was previously shown to behave like a class B GPCR (Simaan, Bedard-Goulet et al. 2005), where it displayed high affinity towards β-arrestin and was retained in endosomes with it.

It should be noted that β-arrestin$_2$ does exhibit early and transient translocation to B2RV2CT6A, even though it was not clearly seen in *figure 11D*. In an unpublished study done in our laboratory, β-arrestin$_2$ tagged with GFP was seen to translocate after about 2 minutes of BK stimulation to untagged B2RV2CT6A receptors at the cell surface, but it did not internalize with B2RV2CT6A in endosomes[1].

With regards to ERK1/2 activation, the temporal patterns observed seemed to correlate with the avidity of the receptor for β-arrestin. In the case of the B2RV2CT6A mutant, the late phase of ERK1/2 activation was less than that of B2RV2CT, and by 60 minutes of BK stimulation it was also less than that of wild-type B2R (at a basal level of

[1] Personal communication, Dr. May Simaan

1%). Because the B2RV2CT6A mutant does not stably bind β-arrestin, the implications could be loss of β-arrestin scaffolding of the ERK1/2 cascade and, subsequently, a blunted late phase of ERK1/2 activation.

The ERK1/2 activation by B2RV2CT was higher than for the wild-type B2R at all stimulation time points. It was shown in the past that the stability of β-arrestin binding determines the extent of ERK activation (Tohgo, Choy et al. 2003). This study found that the angiotensin II type 1a receptor ($AT_{1a}R$) and vasopressin V2 receptor, which are class B GPCRs, activate ERK2 more efficiently than the class A α_{1b}- and β_2-adrenergic receptors. Since B2RV2CT binds β-arrestin with greater avidity than the wild-type B2R does (Simaan, Bedard-Goulet et al. 2005), it is possible that the higher long-term activation observed for the B2RV2CT chimera, compared to wild-type receptor, was due to prolonged β-arrestin scaffolding of the ERK1/2 cascade.

The exception to all the above would be the B2R5A mutant: it did not exhibit any β-arrestin binding, and we propose that its highly sustained activation of ERK1/2 is due to a lack of desensitization and/or an impaired internalization. As B2R5A does not bind β-arrestin, there should be no β-arrestin scaffolding effect present with long-term BK stimulation. Since the serine/threonine cluster that was mutated in B2R has been previously established to be a phosphorylation (desensitization) target as well as an important determinant for internalization (Pizard, Blaukat et al. 1999), this mutant's signaling pathways may be deregulated. It could be that the negative feedback mechanisms that normally dampen the wild-type receptor's response are absent in this case. With the vast majority of the B2R5A receptors trapped at the plasma membrane and not efficiently desensitized and/or internalized, G protein activation probably occurred unchecked in a prolonged manner, and the sustained ERK1/2 activation that resulted may

have been due to an overactivation of the G protein-dependent phase that is normally early and transient in GPCRs. In fact, preliminary evidence from our laboratory with a PKC inhibitor exhibited a decrease in the sustained signal of B2R5A when cells were treated with the inhibitor[2]. PKC is a G protein-mediated second messenger that is known to contribute to ERK1/2 activation.

4.2. Preliminary evidence for the existence of a β-arrestin-ERK1/2 scaffold for the bradykinin type 2 receptor

Finally, we have preliminary evidence from this study pointing towards the existence of a β-arrestin-dependent phase of ERK1/2 activation downstream of activated bradykinin type 2 receptor. This evidence came from employing a cell line that was double-null for both β-arrestins 1 and 2. It was apparent that endogenous B2 receptors in the knockout cell line activate ERK1/2 much less than the wild-type cell line (from 5 minutes of BK stimulation and onwards), suggesting that β-arrestin contributes to a long phase of ERK1/2 activation as a result of B2R activation. However, this type of experiment has limitations. Since the two cell lines came from different individual mice, there could be inherent differences in the clones of cells, such as in the number of receptors expressed on the cell surface. Also, the fact that the double-null cell line did not display more activation-sensitive (i.e., less desensitized) B2 receptors could not be explained, given the fact that the β-arrestins were absent and the B2R should have been, in theory, overactive. GPCR kinases (GRKs) or second-messenger kinases (PKA and PKC) may have been sufficient for desensitization of the receptor in this case, but these results should be verified by another method such as siRNA. With the siRNA approach, the same cell line used for the other experiments (HEK293) would be subjected to RNA interference so as

[2] Personal communication, Dr. Marie-Yvonne Akoume

to knock down expression of both β-arrestin 1 and 2, in order to study what effect their loss would have on ERK1/2 activation following stimulation of exogenously-expressed B2 receptor.

Another approach would be to exogenously express β-arrestin 1 and 2 in the β-arrestin-null MEF cell line in order to study whether this exogenous β-arrestin expression can rescue the blunted late phase of ERK1/2 activation. We have previously attempted this using lipid-mediated transfection of the MEF cell line, but the levels of β-arrestin expression were too low to observe any changes (data not shown). We are currently developing an electroporation technique that would allow more efficient transfection of the β-arrestin-null MEF cell line.

It would also be interesting to see what effect the truncated form of β-arrestin (β-arrestin$_2$T381) would have on the ERK1/2 activation patterns of both the wild-type B2R and the mutant B2R5A. Confocal microscopy studies done here and in the past by our laboratory have revealed that this truncated form of β-arrestin$_2$ readily binds wild-type B2R (Simaan, Bedard-Goulet et al. 2005; Gousseva, Simaan et al. 2008). However, we found that it is unable to bind the mutant B2R5A. This indicates that the serine/threonine cluster in the C-terminal region of the B2 receptor is important for binding to any form of β-arrestin, including this truncated form. We expect that β-arrestin$_2$T381 may not have an effect on the sustained ERK activation by B2R5A, since we have shown that it does not bind to the mutant receptor. However, it may have an effect on the wild-type B2 receptor. It is very plausible that the temporal pattern of ERK1/2 activation by the wild-type receptor would be subsequently modified, most likely decreased. If a decrease in ERK1/2 activation downstream of the wild-type receptor is observed, then β-arrestin$_2$T381 would be likely promoting more efficient desensitization of B2R than wild-type β-arrestin$_2$.

Also, another possible explanation for such a result may be that β-arrestin$_2$T381 is missing regions that are important for scaffolding the various components of the ERK1/2 pathway, and would thus not be able to facilitate B2R-mediated ERK1/2 activation as robustly as wild-type β-arrestin$_2$.

4.3. EGF receptor transactivation

Many studies have emerged over the past decade that point to the existence of interaction between GPCRs and the epidermal growth factor receptor (EGFR) (Gschwind, Zwick et al. 2001). The EGFR, a receptor tyrosine kinase with a mitogenic pathway, has been found to be activated by GPCRs via ligand-dependent and ligand-independent manners. The first evidence hinting that the EGFR is a downstream target of GPCRs came from a study that employed Rat-1 fibroblasts and the GPCR ligands lysophosphatidic acid, thrombin and endothelin-1. It was found that stimulating the cells with these GPCR agonists resulted in phosphorylation of tyrosine residues on the EGF receptor (the mechanism by which the receptor becomes activated) (Daub, Weiss et al. 1996). It also became apparent that in some cases, there is a role for the tyrosine kinase Src, which is recruited to activated GPCR via β-arrestin (Luttrell, Ferguson et al. 1999), and which phosphorylates EGFR at the tyrosine residue 845 (Biscardi, Maa et al. 1999). Some GPCR agonists have been found to induce phosphorylation of this putative Src phosphorylation site (Santiskulvong and Rozengurt 2003). It has been proposed that one way that GPCRs activate the mitogenic MAPK pathway is via transactivation of the EGF receptor, in a Src-dependent or –independent manner, and that this activation of EGFR leads to the activation of Ras/Raf/MEK/ERK (Luttrell, Daaka et al. 1999).

Bradykinin is known to induce tyrosine phosphorylation of EGFR, leading to MAPK activation (Zwick, Daub et al. 1997; Mukhin, Garnovsky et al. 2003; Greco, Muscella et

al. 2004), and that this may occur via Src (Hur, Park et al. 2004). Interestingly, bradykinin has been shown to transinactivate, rather than activate, EGFR in some cases (Graness, Hanke et al. 2000). It would be worthwhile to investigate the role of EGFR, if any, in β-arrestin-dependent ERK1/2 activation downstream of activated bradykinin type 2 receptor. This could be done by employing one of the commercially-available pharmacological inhibitors of the EGF receptor, and observing any changes to the temporal patterns of B2R-mediated ERK1/2 activation. Similar experiments could be performed with a pharmacological inhibitor of Src, since this non-receptor tyrosine kinase seems to sometime act as a link between activated GPCRs and the EGF receptor. It is plausible that the kinetics of ERK1/2 activation via wild-type B2 receptor may be modulated with the use of such inhibitors, but their effects, if any, on the B2R5A mutant are less predictable. We have proposed that B2R5A has very weak or non-existent interaction with β-arrestin and that the highly sustained ERK1/2 phosphorylation is likely due to overaction of G proteins. Thus, we expect that pharmacological inhibitors of EGFR and Src are likely to modulate ERK1/2 activation downstream of wild-type B2 receptor, but that their effects on the B2R5A mutant may be less robust.

4.4. Contribution of the present study towards understanding modes of MAP kinase activation and their biological significance in the cell and in pathophysiological states

It has been proposed that the cellular localization of activated ERK1/2 downstream of GPCRs differs depending on whether ERK1/2 activation is rapid and transient, or late and more sustained. The angiotensin II type 1A receptor ($AT_{1A}R$) activates ERK1/2 in a G protein-dependent manner (likely G_q/G_{11}-dependent) and in a β-arrestin-dependent manner. It was found that the G protein-dependent phase of ERK1/2 activation occurs

rapidly and is short lived (lasting about 2 minutes), while the β-arrestin-dependent phase is delayed (occurring 5-10 minutes following Ang II stimulation) but persists for much longer (up to 90 minutes). A pool of ERK1/2 activated via the G protein was found to translocate to the nucleus, whereas ERK1/2 activated via β-arrestin (late phase) was found to remain in the cytoplasm (Ahn, Shenoy et al. 2004). Thus, β-arrestin desensitizes the G protein-dependent, first wave of ERK1/2 activation, but also scaffolds components of the ERK pathway resulting in a more persistent, second wave of ERK1/2 activation.

These differences in the spatial localization of activated ERK1/2 may have considerable physiological effects. In fact, it has been shown for the $AT_{1A}R$ and the vasopressin V2 receptor that overexpression of β-arrestin 2 results in retention of more activated ERK1/2 in the cytoplasm, with a decrease in activation of nuclear transcription factor targets of ERK1/2 (Tohgo, Pierce et al. 2002; Tohgo, Choy et al. 2003). Thus, β-arrestin-mediated ERK1/2 activation may antagonize some of the effects produced by mitogenic signaling, such as cell proliferation. The identities of substrates of activated ERK1/2 retained in the cytoplasm by β-arrestin are unclear.

It would be interesting to investigate whether the same is true for bradykinin type 2 receptor-mediated ERK1/2 activation. If ERK1/2 activation downstream of wild-type B2R does indeed have differential spatial localization in the cell, then it is plausible that receptor mutants might shift the balance towards one or the other. We believe that the B2R5A mutant employed in our study herein highly sustains ERK1/2 activation, compared to wild-type B2R, due to overactivation of the normally transient G protein phase, and that a β-arrestin-dependent second wave of ERK1/2 activation may be negligible, due to the mutant receptor's very weak affinity for β-arrestin. We thus predict that the highly sustained, G protein-dependent activation downstream of the B2R5A

mutant results in an increase in the pool of ERK1/2 translocated to the nucleus, and thus an increase in the activation of transcription factors which modulate genes that are involved in the mitogenic actions of the MAPK pathway.

Since the present study examines bradykinin activation of the ERKs 1 and 2, which are members of the mitogenic family of MAP kinases, these results have relevance to research on cancer, and it may be worthwhile to understand the mechanisms of ERK1/2 activation by the braydkinin type 2 receptor.

The B2 receptor has been shown in previous studies to be expressed in prostate cancer cell lines (Clements and Mukhtar 1997; Srinivasan, Kosaka et al. 2004). In primary cultures of normal human prostate stromal cells, BK stimulation resulted in cellular proliferation as well as activation of ERK1/2. These effects were blocked by the specific B2R antagonist HOE140 (Srinivasan, Kosaka et al. 2004). BK is known to behave as an autocrine growth factor in prostate cancers, and it also promotes tissue permeability and angiogenesis, thereby assisting tumor invasion (Stewart, Chan et al. 2002).

ERK1/2-mediated proliferation via BK stimulation was also observed in primary cultures of breast cancer cells. This effect was blocked with a PLC-β inhibitor, a MAPKK inhibitor, and an inhibitor of non-conventional PKC isoforms (but not with an inhibitor of conventional PKC isoforms) (Greco, Elia et al. 2005). These studies suggest that the bradykinin type 2 receptor may be a viable therapeutic target for prostate and breast cancers.

In addition to its proposed contribution to prostate and breast cancers, BK-stimulated ERK1/2 activation has been suggested as a diagnostic tool for Alzheimer's disease (Khan and Alkon 2006). This study found that basal phosphorylation of ERK1/2 was lower in Alzheimer's disease patients than age-matched controls, and that BK stimulation causes

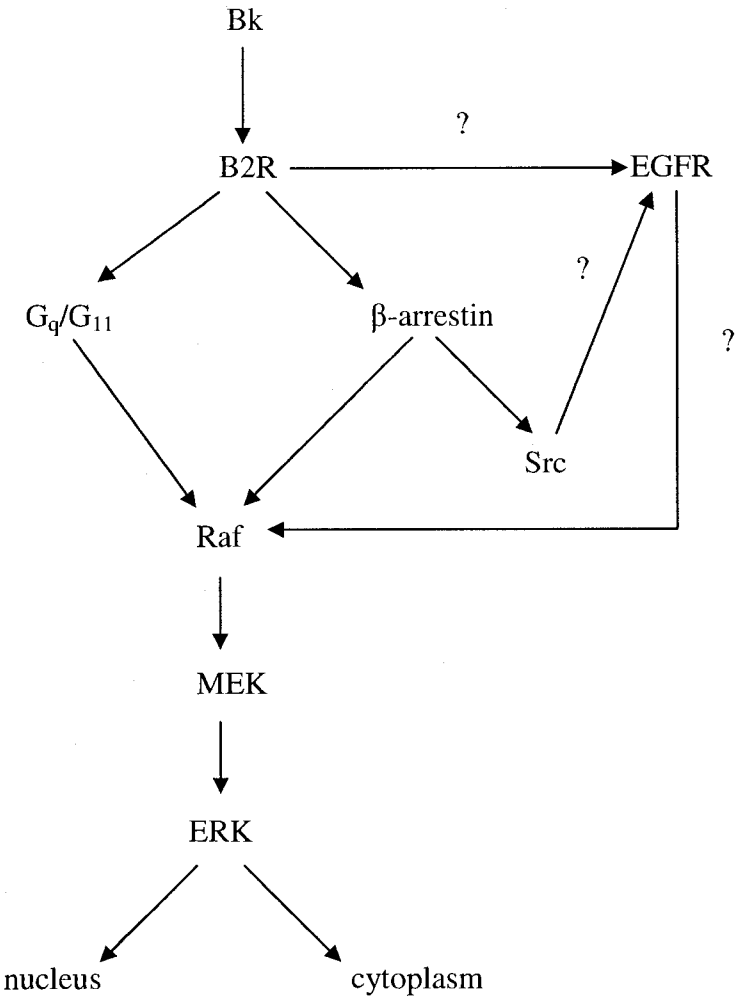
greater phosphorylation of ERK1/2 in patients than in controls. A diagnostic index using phosphorylation of ERK1/2 as a biomarker was proposed, and it was noticed that duration of Alzheimer's disease was correlated with decreased phosphorylation of ERK1/2. Since it is clinically difficult to diagnose early-stage Alzheimer's disease, BK-mediated ERK1/2 activation may be an effective tool for its diagnosis.

The contributions of β-arrestin to these pathologies are as yet unknown. It would be worthwhile to examine deregulation of β-arrestin in these diseases; be it aberrant expression, intracellular targeting or activity, or some other mechanism. The results herein thus allow us a better understanding of β-arrestin-mediated ERK1/2 activation downstream of the bradykinin type 2 receptor, with implications for both normal and patho-physiological states.

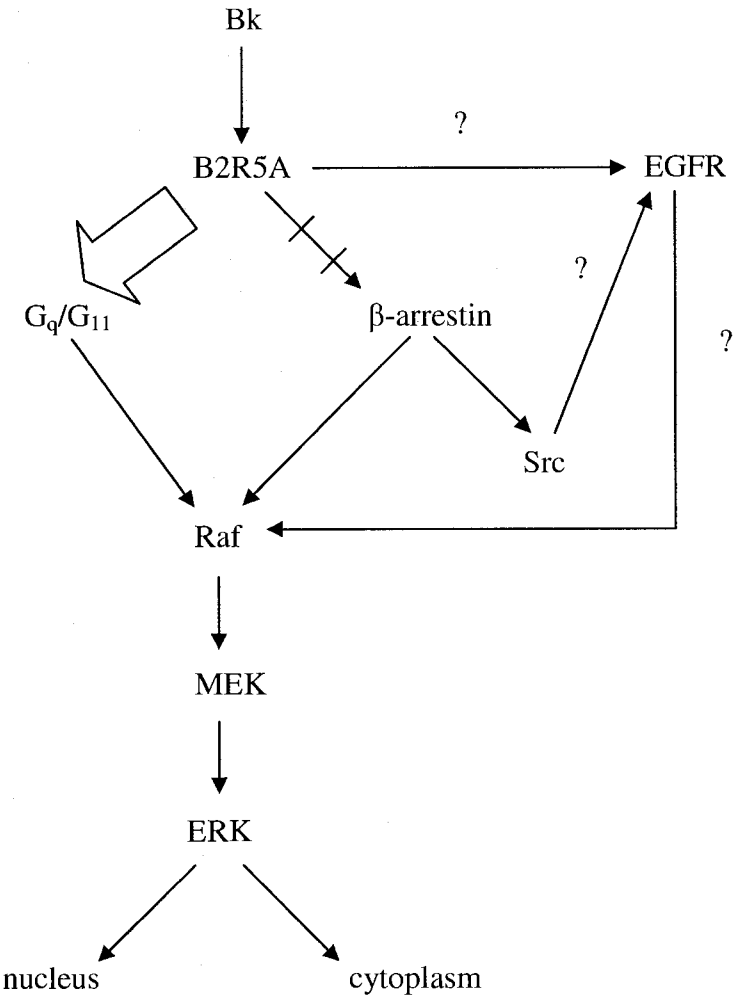
Figure 16 illustrates a model summarizing experimental evidence from this study as well as speculations discussed here.

Figure 16: Models of ERK activation by the wild-type B2 receptor and the mutant B2R5A. *A*, Activation of ERK downstream of the bradykinin B2 receptor. Stimulation of the G_q/G_{11}-coupled B2 receptor with bradykinin results in activation of the ERK pathway. Evidence from our study suggests that β-arrestin can also activate the ERK MAPK pathway, likely via a scaffolding effect. If the B2 receptor has similar properties to the AT_{1A} receptor with regards to the spatial localization of activated ERK, then G protein-activated ERK would translocate to the nucleus, whereas ERK activated via β-arrestin scaffolding would be retained in the cytoplasm. In addition, EGF receptor transactivation could also be playing a role in ERK activation, and this transactivation may occur in a Src-dependent or –independent manner. *B*, Activation of ERK downstream of the mutant B2R5A. In this model, the mutant receptor activates ERK in a highly sustained manner via overactivation of the G protein, with negligible contribution by β-arrestin.

A


Bk
?
B2R
EGFR
?
G_q/G_{11}
β-arrestin
?
Src
Raf
MEK
ERK
nucleus
cytoplasm

B


Bk
?
B2R5A
EGFR
?
G_q/G_{11}
β-arrestin
?
Src
Raf
MEK
ERK
nucleus
cytoplasm

4.5. Conclusions

To summarize the results of the present study:

1. The ERK1/2 activation by the bradykinin type 2 receptor likely has a β-arrestin-dependent component;

2. Changing the avidity of B2R to β-arrestin correlates with the extent of ERK1/2 activation;

3. The serine/threonine cluster in the C-terminal tail of the B2R is important for receptor desensitization and internalization, with mutations causing highly sustained ERK1/2 activation in a deregulated manner. In constrast, mutations in a comparable cluster in the vasopressin C-terminus of the B2RV2CT chimera result in decreased ERK activation, indicating that serine/threonine clusters do not play identical functions in the bradykinin B2 and vasopressin V2 receptors.

5. Bibliography

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