Cellular Signalling 23 (2011) 648–659

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Role of ßarrestins in bradykinin B2 receptor-mediated signalling

Brandon Zimmerman [a,b], May Simaan [a], Marie-Yvonne Akoume [a], Nadia Houri [a], Stéphanie Chevallier [c], Philippe Séguéla [c], Stéphane A. Laporte [a,b,*]

[a] McGill University Health Center Research Institute, Department of Medicine, Royal Victoria Hospital, QC, H3A 1A1, Canada
[b] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, H3G 1Y6, Canada
[c] Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, H3A 2B4, Canada

ARTICLE INFO

Article history:
Received 28 September 2010
Received in revised form 22 November 2010
Accepted 25 November 2010
Available online 8 December 2010

Keywords:
G protein-coupled receptor
Bradykinin B2 receptor
ßarrestin
Extracellular signal-regulated kinase 1/2

ABSTRACT

G protein-coupled receptors (GPCRs) can engage multiple pathways to activate ERK1/2 via both G proteins and/or ßarrestin. Receptor recruitment of ßarrestin is also important for GPCR desensitization, internalization and resensitization. Modulation of the receptor/ßarrestin interaction through modification of either component would presumably alter the output generated by receptor activation. Here we examined how ßarrestins regulate bradykinin (BK) B2 receptor (B2R) signalling and desensitization by either truncating ßarrestin1 or ßarrestin2 or by alanine substitution of a serine/threonine cluster in the C-terminal tail of B2R (B2R-4A), conditions which all affect the avidity of the B2R/ßarrestin complex. We first demonstrate that BK-mediated ERK1/2 activation is biphasic containing an early peak (between 2–5 min) followed by sustained activation for at least 60 min. The early but not the sustained phase was predictably affected by inhibition of either Gαq/11 or Gαi/o, whereas loss of ßarrestin2 but not ßarrestin1 resulted in diminished prolonged ERK1/2 activation. ßarrestin2's role was further examined using a truncation mutant with augmented avidity for the agonist-occupied receptor, revealing an increase in both immediate and extended ERK1/2 signalling. We also show that ERK1/2 is recruited to the B2R/ßarrestin complex on endosomes as well as the plasma membrane. Moreover, we investigated ßarrestin's role using the B2R-4A, which is deficient in ßarrestin binding and does not internalize. We show that ERK1/2 signalling downstream of the receptor is entirely G protein-dependent and receptor-mediated intracellular calcium mobilization studies revealed a lack of desensitization. Functionally, the lack of desensitization resulted in increased cell growth and migration compared to the wild-type receptor, which was sensitive to MEK inhibition. These results highlight ßarrestin's crucial role in the maintenance of proper B2R signalling.

1. Introduction

The mitogen-activated protein kinase (MAPK) family members are important regulators for biological processes such as cell growth, differentiation and survival with deregulation of MAPK pathways potentially leading to adverse physiological consequences. G protein-coupled receptors (GPCRs) employ a myriad of distinct mechanisms to activate MAPK family members including the extracellular signal-regulated protein kinases (ERK1/2) in a receptor- and cell type-dependent fashion [1,2]. GPCR-dependent ERK1/2 activation was originally predicated based on the involvement of G protein-dependent signalling through protein kinase A (PKA) and PKC [3] and cross-talk with classical receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) [4,5] or the platelet-derived growth factor receptor (PDGFR) [6,7]. More recently, it was established that GPCRs engage the ERK1/2 pathway through the scaffolding functions of ßarrestins, independent from the functional activation of G protein [8].

Beta-arrestins are adaptors that were originally described for their role in homologous desensitization of GPCRs. Upon GPCR binding, they serve to functionally uncouple the G proteins from the receptor, mitigating the loss of second-messenger signals. Subsequently, the desensitized GPCRs are targeted for receptor endocytosis via clathrin-coated pits [9]. The process of desensitization and endocytosis is critical for proper receptor signalling and function, and disruptions to these processes lead to aberrant physiological outcomes. As mentioned above, ßarrestins have recently emerged as signalling adaptors linking receptors to multiple downstream effectors such as MAPK family members (ERK1/2, p38 and JNK), Src kinase, Akt, PI3 kinase and RhoA [8]. Their ability to activate the ERK1/2 pathway has been demonstrated downstream of numerous GPCRs including but not

Abbreviations: GPCRs, G protein-coupled receptors; ERK1/2, extracellular signal-regulated protein kinases; BK, bradykinin; B2R, bradykinin type 2 receptor; B2R-4A, B2R where 4 serine and threonine were mutated to alanines (Thr342/345, Ser346/348); GRK, G protein receptor kinase; MAPK, mitogen-activated protein kinase; ß2AR, ß2 adrenergic receptor; AT1R, angiotensin II type 1 receptor; NK-1R, neurokinin-1 receptor; V2R, vasopressin type 2 receptor; PTH1R, parathyroid hormone receptor; YFP, yellow fluorescent protein; PAR-2, protease-activated receptor; PKC, protein kinase C; mRFP, monomeric red fluorescent protein.

* Corresponding author. 687 Pine Avenue West, Room H7.83, Royal Victoria Hospital, Montreal, Quebec, H3A 1A1, Canada. Tel.: +1 514 934 1934x36272; fax: +1 514 982 0893.
E-mail address: stephane.laporte@mcgill.ca (S.A. Laporte).

0898-6568/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2010.11.016

limited to ß2AR [10], AT1R [11,12], neurokinin-1 receptor (NK-1R) [13], vasopressin type 2 receptor (V2R) [14], parathyroid hormone receptor (PTH1R) [15] and protease-activated receptor (PAR-2) [16]. Furthermore, it was also proposed that ßarrestin-mediated ERK1/2 activation is determined by the stability of the interaction between receptors and ßarrestins. Hence, Class B receptors that form stable complexes with ßarrestin on endosomes activate ßarrestin-bound ERK1/2 more efficiently than class A receptors, which rapidly dissociate from ßarrestin at the plasma membrane following receptor internalization [17]. However, less is known about the contribution of ßarrestin on MAPK activation for Class C receptors such as the bradykinin (BK) B2 receptor (B2R), which internalize with ßarrestin into endosomes, but where the complex is more labile than class B GPCRs [18]. Within the C-terminal tail of B2R, there exists a serine/threonine cluster that has been reported to be a key determinant for its phosphorylation, desensitization and internalization [19,20]. Moreover, we have recently shown that this region of the B2R is also important for binding ßarrestin and regulating the resensitization of the receptor [18]. The stability of the ßarrestin/receptor complex is in part regulated by the phosphorylation of this serine/threonine cluster [21]. While these findings highlight the importance of the B2R C-terminal tail for ßarrestin binding, the extent to which ßarrestin regulates B2R-mediated ERK1/2 responses in cells remains ill-defined.

BK is a key regulator of vascular tone that has been implicated in pathological disorders such as inflammation, pain and cancer (for review see [22]). It acts through two distinct GPCRs, the pathologically-induced B1R and the constitutively expressed B2R [23]. BK-stimulation of B2R leads to activation of the Gαq/11 family of heterotrimeric G proteins in many cell types [22], which in turn stimulates the activity of phosphatidylinositol-specific phospholipase C (PLC) to yield PI hydrolysis and increase intracellular Ca^{2+}. Although predominantly described to couple to Gαq/11, the B2R has also been reported to activate pertussis toxin-sensitive Gαi/o [24]. B2R-mediated Gαq/11 and/or Gαi/o activation has been shown to be involved in many cellular responses including the mitogenic response through activation of ERK1/2 [24,25] and/or cross-talk with EGFR [26,27].

Despite its relative high avidity for ßarrestin, the B2R/ßarrestin complex dissociates rapidly in endosomes, and the receptor recycles to the plasma membrane following agonist removal [18]. Based on recent evidence suggesting that the avidity of the GPCR/ßarrestin complex contributes in the regulation of temporal and spatial MAPK responses, the present study was pursued to reveal the role of ßarrestins in B2R-mediated ERK1/2 activation and mitogenic responses in cells.

2. Materials and methods

2.1. Materials

The monoclonal anti-Myc antibody, bradykinin and pertussis toxin were from Sigma Chemical Co. The inhibitors Gö6983 and PD98059 were from Calbiochem. DMSO was from Bioshop. Antibodies against phospho-ERK1/2 and total ERK1/2 were from Cell Signalling Technology. The polyclonal antibody against the C-terminal domain of ßarrestins, BARR3978 was described elsewhere [28]. Anti-HA antibodies coupled to agarose beads and mouse anti-HA (clone 12CA5) were purchased from Roche. Fura-2-AM was purchased from Molecular Probes. Anti-mouse and anti-rabbit HRP-conjugated IgG were from Sigma and the enhanced chemiluminescence lightening (ECL) was obtained from Perkin-Elmer. Alexa fluor 568 anti-rabbit and Alexa fluor 488 anti-mouse secondary antibodies were purchased from Molecular Probes. ShRNA against ßarrestin1 was purchased from Open Biosystems and shRNA against ßarrestin2 and control shRNA were a gift from Dr. Marc Caron (Duke University, Durham, NC). mRFP in pRSET was a gift from Dr. Roger Tsien (University of California at San Diego, La Jolla, CA).

2.2. Plasmids and constructs

Plasmids encoding B2R, HA-B2R, ßarrestin2-YFP, ßarrestin2-381X-YFP, ßarrestin1-YFP, ßarrestin1-383X-YFP and B2R-YFP were described elsewhere [18,21,29]. B2R-4A was generated by PCR using a forward primer overlapping a *ClaI* site and a reverse primer overlapping an *XbaI* site in B2R (pcDNA3). The reverse primer contained alanine mutations as substitutions for Thr^{342}, Thr^{345}, Ser^{346} and Ser^{348}. The generated PCR fragment was ligated into pcDNA3-B2R cut with *ClaI* and *XbaI*. HA-B2R-4A was generated by excising the *Hind*III/*BspEI* fragment from HA-B2R (pcDNA3) and cloned into B2R-4A in pcDNA3 cut with the same restriction enzymes. Myc-tagged ßarrestin constructs (myc-ßarrestin2, myc-ßarrestin2-381T, myc-ßarrestin1 and myc-ßarrestin1-383T) were cloned SalI/ApaI into pCMV-tag3T-2A in frame with the myc tag. ßarrestin2-mRFP was cloned HindIII/KpnI in pcDNA3.1 containing mRFP tag at the C-terminus. B2R-4A-YFP was generated by PCR. Briefly, a fragment was amplified from B2R-4A cDNA with a forward primer overlapping the *Cla*I site and a reverse primer removing the stop codon and introducing a *Sal*I restriction site. B2R was cut with *Hind*III/*Cla*I from pcDNA3 and the generated fragment was three-way ligated with the digested PCR product into pEYFP-N1 cut with *Hind*III and *Sal*I. All constructs were verified by DNA sequencing (Service d'Analyse et de Synthèse d'Acides Nucléiques, Université Laval, QC, Canada).

2.3. Cell culture and transfection

HEK293 cells were grown in MEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco) and 100 μg/mL gentamicin (Gibco). Cells were seeded in six well plates (1.5×10^5 cells/well), 35 mm glass-bottomed culture dishes (Mattek Corp.) (1.2×10^5 cells/dish) or 10 cm dishes (1.5×10^6 cells/dish). Transfections were performed by a calcium phosphate co-precipitation method as previously described [18]. For calcium imaging, HEK293 cells were grown to 60–80% confluence before transient transfection in 60-mm dishes. The transfection was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The day after, transfected cells were plated on 35 mm poly-D-lysine-coated glass-bottom dishes at 10^5 cells/well. For shRNA transfection, HEK293 cells (2×10^5 cells/well) were transfected for 72 h with HA-B2R (1 μg/well) and either control, ßarrestin1 or ßarrestin2 shRNAs (0.75 μg/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations (Gibco).

2.4. ERK1/2 phosphorylation

HEK293 cells seeded in a 6-well plate at a density of 1.5×10^5 cells/well were transfected with B2R or B2R-4A or HA-B2R and either myc-ßarrestin2, myc-ßarrestin2-381T, myc-ßarrestin1 or myc-ßarrestin1-383T. 36 h post-transfection, cells were serum starved for 2 h in MEM (20 mM HEPES) and then left untreated or treated with BK (1 μM) for the indicated time points. For the experiments done with the pharmacological inhibitors, cells, transfected with HA-B2R, were first treated with the vehicle (DMSO), PKC inhibitor Gö6983 (5 μM) for 1 h or PTX (50 ng/mL) for 16 h before being subjected to BK stimulation. Cells were then lysed in 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, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 2.5 μg/ml aprotinin, 1 mM pepstatin, and clarified by centrifugation. An aliquot of the cell lysates was mixed with an equal volume of Laemmli buffer (250 mM Tris–HCl pH 6.8, 2% SDS (wt/vol), 10% glycerol (vol/vol), 0.01% bromophenol blue (wt/vol), and 5% β-mercaptoethanol (vol/vol)). Lysates were resolved on a SDS-PAGE and blotted for phospho-ERK1/2, total ERK1/2 and myc-ßarrestin.