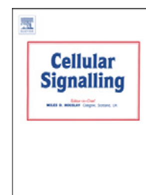
Cellular Signalling 26 (2014) 1523–1531

Contents lists available at ScienceDirect

Cellular Signalling

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



Mutations in arrestin-3 differentially affect binding to neuropeptide Y receptor subtypes

Luis E. Gimenez [a], Stefanie Babilon [b], Lizzy Wanka [b], Annette G. Beck-Sickinger [b], Vsevolod V. Gurevich [a,*]

[a] *Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA*
[b] *Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Leipzig University, Brüderstraße 34, D-04103 Leipzig, Germany*

ARTICLE INFO

Article history:
Received 31 January 2014
Accepted 16 March 2014
Available online 29 March 2014

Keywords:
Arrestins
GPCRs
Neuropeptide Y receptors
Protein engineering
Signal transduction
Bioluminescence resonance energy transfer (BRET)

ABSTRACT

Based on the identification of residues that determine receptor selectivity in arrestins and the phylogenetic analysis of the arrestin (arr) family, we introduced fifteen mutations of receptor-discriminator residues in arr-3, which were identified previously using mutagenesis, in vitro binding, and BRET-based recruitment assay in intact cells. The effects of these mutations were tested using neuropeptide Y receptors Y1R and Y2R. NPY-elicited arr-3 recruitment to Y1R was not affected by these mutations, or even alanine substitution of all ten residues (arr-3-NCA), which prevented arr-3 binding to other receptors tested so far. However, NCA and two other mutations prevented agonist-independent arr-3 pre-docking to Y1R. In contrast, eight out of 15 mutations significantly reduced agonist-dependent arr-3 recruitment to Y2R. NCA eliminated arr-3 binding to active Y2R, whereas Tyr239Thr reduced it ~7-fold. Thus, manipulation of key residues on the receptor-binding surface generates arr-3 with high preference for Y1R over Y2R. Several mutations differentially affect arr-3 pre-docking and agonist-induced recruitment. Thus, arr-3 recruitment to the receptor involves several mechanistically distinct steps. Targeted mutagenesis can fine-tune arrestins directing them to specific receptors and particular activation states of the same receptor.

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1. Introduction

G protein-coupled receptors (GPCRs) are high value drug targets, with almost half of the marketed drugs acting on these cell surface proteins [1,2]. The demand for specific tools for molecular intervention keeps growing, with the emphasis on minimizing side effects and expanding the repertoire of therapeutically addressable GPCRs. The identification of arrestins as key regulators in GPCR desensitization and internalization and mediators of G protein-independent signaling opened new possibilities for the manipulation of receptor function. Arrestins bind activated and phosphorylated GPCRs and block receptor–G-protein interaction, while serving as adaptors for key components of the endocytic machinery and numerous signaling proteins [3,4]. The two visual subtypes, arrestin-1[1] (arr-1) and arrestin-4 (arr-4), are expressed exclusively in photoreceptors and pinealocytes, and bind rhodopsin and cone opsins [5,6]. In contrast, two ubiquitously expressed non-visual subtypes, arrestin-2 (arr-2) and arrestin-3 (arr-3), are fairly promiscuous and interact with a huge range of GPCRs [3]. Recent advances in our understanding of the molecular basis of receptor specificity of arrestin proteins pave the way to targeted construction of reengineered receptor-specific arrestins [7,8].

Here we tested the interactions of mutants of the non-visual arr-3 and two members of the neuropeptide Y (NPY) receptor family. The NPY system plays a central role in the regulation of energy homeostasis and is involved in the pathophysiology of cancer progression, obesity, mood disorders, and epilepsy [9]. This makes the neuropeptide Y system a desirable drug target for future therapies. NPY receptors respond to three endogenous peptidic agonists, NPY, peptide YY (PYY) and the pancreatic polypeptide (PP). Neuropeptide Y receptor family includes four subtypes in humans: Y1R, Y2R, Y4R, and Y5R [10]. Y receptors belong to class A of the GPCR superfamily, coupled to $G_{i/o}$ proteins [11], and are expressed in numerous tissues including the brain, blood

Abbreviations: GPCR, G protein-coupled receptor; arr, arrestin; NPY, neuropeptide Y; Y1R, neuropeptide Y1 receptor; Y2R, neuropeptide Y2 receptor; BRET, bioluminescence resonance energy transfer; ANOVA, analysis of variance; arr-3-K2A, arrestin-3-Lys11 AlaLys12Ala; arr-3-NCA, arrestin-3 Leu49Ala, Asp51Ala, Arg52Ala, Leu69Ala, Tyr239Ala, Asp241Ala, Cys252Ala, Pro253Ala, Asp260Ala, and Gln262Ala; arr-3-KNC, arrestin-3 Lys11Ala Lys12Ala, Leu49Ala, Asp51Ala, Arg52Ala, Leu69Ala, Tyr239Ala, Asp241Ala, Cys252Ala, Pro253Ala, Asp260Ala, and Gln262Ala; REGCP, arrestin-3 Asp260-Gln262*delins*Arg Glu, Gly, Cys, Pro; NEGFP, arrestin-3 Asp260-Gln262*delins*Asn Glu, Gly, Phe, Pro.

* Corresponding author at: Department of Pharmacology, Vanderbilt University, 2220 Pierce Ave., Preston Research Building, Rm 417D, Nashville, TN 37232, USA. Tel.: +1 615 322 7070; fax: +1 615 343 6532.
E-mail address: vsevolod.gurevich@vanderbilt.edu (V.V. Gurevich).

[1] We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48-kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons, its gene is called “arrestin 3” in the HUGO database).

http://dx.doi.org/10.1016/j.cellsig.2014.03.019
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vessels, heart, and gastrointestinal tract [12–16]. They have overlapping binding profiles for their endogenous ligands with Y1R, Y2R, and Y5R binding NPY and PYY with high affinity, whereas Y4R prefers PP [17].

In this complex multiligand–multireceptor system Y1R and Y2R occupy a distinct position. They are frequently expressed in the same tissues [18], where they can induce either synergistic or antagonizing effects on food intake, anxiety, and depression [19,20]. Antagonism is thought to be due to the pre-synaptic expression of Y2R in NPY-ergic neurons, where it functions as an inhibitory autoreceptor blocking NPY release and thus Y1R- and Y5R-mediated effects [21], as revealed by using Y1R- and Y2R-selective agonists and antagonists. However, as Y receptor knockout models showed inconclusive results [20,22], subtype selective arrestins would be valuable tools to unravel phenotypes assigned to either Y1R- or Y2R-mediated effects.

Unlike the Y5R, both Y1R and Y2R undergo arr-3-dependent internalization, which terminates their G-protein-mediated signaling. Arr-3 binding motifs in these receptors have been recently identified. Like in many GPCRs, these motifs include C-terminal serine/threonine clusters which serve as phosphorylation substrates [23–25] for G protein-coupled receptor kinases (GRKs [26]). Even though phosphorylated serines and threonines favor the interaction of non-visual arrestins with their cognate receptors, previous studies showed that phosphorylation does not contribute to the arrestin receptor specificity and is not even mandatory for all arrestin–GPCR interactions [27–29]. Although arrestins engage an extensive surface of the receptor, only a few residues on the concave sides of the arrestin N- and C-domains determine its receptor specificity [28]. This finding made it possible to construct the mutants of naturally promiscuous arr-3 that are specific for GPCR groups or single receptor subtypes [30]. Importantly, these studies on D1 and D2 dopamine, β_2-adrenergic (β2AR) and M2 muscarinic receptors showed that mutations of arr-3 similarly affected the interactions with activated phosphorylated receptors and agonist-independent arrestin pre-docking [30]. This supports the idea that arrestins preselect their target receptors before they become active and phosphorylated, enabling the use of reengineered arrestins for the manipulation of GPCR functions. Here we report the identification of key arr-3 residues that discriminate between Y1 and Y2 receptors and their functional states.

2. Materials and methods

2.1. Materials

Porcine NPY (pNPY) was produced by automated solid phase peptide synthesis using the Fmoc/tBu (9-fluorenylmethoxycarbonyl-tert-butyl) strategy, as described [31]. [^{3}H] *myo*-inositol was from GE Healthcare Europe GmbH (Braunschweig, Germany). Restriction endonucleases and other DNA modifying enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech-Corning (Manassas, VA), Life-technologies (Carlsbad, CA), or PAA Laboratories GmbH (Pasching, Austria). Luciferase substrate coelenterazine-*h* was from NanoLight Technology (Pinetop, AZ). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich (St Louis, MO).

2.2. Mutagenesis and plasmid construction

Plasmids encoding short splice variant of bovine arr-3 [32,33] with unique restriction sites introduced by silent mutations and arr-3 mutants K2A, NCA and KNC with secondary phosphate-sensing N-terminal lysines (Lys11 and Lys12) or all key receptor-discriminator residues replaced with alanines, were described previously [27,28]. All mutations were introduced on Ala87Val background, as described [7,27,30], which mimics more receptor-specific visual subtypes [34,35], reducing the flexibility of the N-domain [36,37]. To generate all mutants, mutagenizing oligonucleotides were used as forward primers and an oligonucleotide downstream from the far restriction site to be used for subcloning was used as a reverse primer. Resulting fragments of various lengths and an appropriate primer upstream of the near restriction site were then used as reverse and forward primers, respectively, for the second round of PCR. Restriction sites Bam HI and Bsi WI were used for introducing mutations in the N-domain, and sites Age I and Xho I were used for introducing mutations in the C-domain. The resulting fragments were purified, digested with the respective enzymes, and subcloned into the suitably digested pGEM-2 plasmid (Promega; Madison, WI) containing the sequence of wild type (WT) bovine arr-3 [38,39]. For bioluminescence resonance energy transfer (BRET) assays, WT and mutant arr-3 were subcloned into pcDNA3 Venus plasmid (Life technologies), yielding arr-3 N-terminally tagged with Venus [40]. Human Y1R and Y2R were C-terminally tagged with *Renilla* luciferase variant 8 (*R*Luc8 [41]), as described [27,28,30]. All constructs were verified by dideoxy sequencing.

2.3. Functional characterization of *R*Luc-tagged Y receptors

Signaling of *R*Luc-tagged Y1R and Y2R was measured in transiently transfected COS-7 cells (ATCC Cat. # CRL-1651) by determining the inositol phosphate (IP) accumulation after pNPY stimulation, as described [42,43], using the method established by Berridge [44,45]. Cells were treated with increasing concentrations of pNPY, from 10 pM to 1 μM. To determine EC_{50} values for pNPY on Y1R and Y2R, the data were fit to a three-parameter dose–response curve equation (GraphPad Prism 6.03).

2.4. Bioluminescence resonance energy transfer (BRET) assay

BRET-based [46,47] arrestin–receptor interaction assays were performed and analyzed, as described [24,27,30,48]. Cells were treated with pNPY (1 μM) for 8 min prior to the addition of 5 μM coelenterazine-*h*. Absolute levels of luminescence were used as the measure of the expression levels of RLuc-tagged receptors, whereas direct fluorescence was used to gauge the expression of Venus-tagged arrestins, as described [24,27,30,37]. BRET was measured 15 min after agonist addition, as described [24], and plotted as a function of fluorescence/luminescence ratio. The resulting curves were fitted by a non-linear regression to a one-site hyperbola equation (net BRET after pNPY treatment) or a three-parameter dose–response curve equation (BRET ratios vs fluorescence/luminescence (F/L) ratios for each arrestin amount) (GraphPad Prism 6.03).

2.5. Visualization of arrestin recruitment to the receptor by live-cell imaging

COS-7 cells were plated onto sterile 8-well μ-slides (ibidi GmbH, Martinsried/Munich, Germany), grown to 70% confluence and transfected with 1 μg of DNA encoding Y1R or Y2R fused to eCFP along with 0.1 μg of DNA encoding WT Venus-arr-3, or Ala87Val, Ala87Val + Tyr239Thr, and KNC mutants, using Lipofectamine™ 2000 (Life technologies). Imaging was performed 24 h after transfection using a Zeiss Axio Observer microscope with an ApoTome Imaging System equipped with a Heating Insert P Lab-Tek S1 unit. Fluorescence images from living cells were taken at 37 °C on a heated microscope stage using the AxioVision Rel. 4.6 software. Arr-3 translocation was visualized in serum-starved cells for 30 min in Opti-MEM® (Life technologies) and treated with 1 μM pNPY at 37 °C for 15 min. Images (EYFP/Venus channel only) were acquired before and after agonist treatment.

2.6. Data analysis and statistics

Statistical analysis was performed using Prism 6.03 (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way ANOVA followed by post-hoc Dunnett's test with correction for multiple comparisons.

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