Cellular Signalling 24 (2012) 991–1001

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

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

Heterodimerization of human apelin and kappa opioid receptors: Roles in signal transduction

Yalin Li [a,b,1], Jing Chen [c], Bo Bai [c,*], Hui Du [a,b], Youwang Liu [b], Haiqing Liu [b]

[a] School of life science, Shandong Agricultural University, Taian, 271018, China
[b] Department of Neurobiology, Taishan Medical University, Taian, 271000, China
[c] Department of Neurobiology, Jining Medical University, Jining, 272067, China

ARTICLE INFO

Article history:
Received 17 August 2011
Received in revised form 11 December 2011
Accepted 12 December 2011
Available online 20 December 2011

Keywords:
Apelin receptor
Kappa opioid receptor
GPCRs
Heterodimerization
Signal transduction

ABSTRACT

Apelin receptor (APJ) and kappa opioid receptor (KOR) are members of the family A of G protein-coupled receptors (GPCRs). These two receptors are involved in the central nervous system regulation of the cardiovascular system. Here, we explore the possibility of heterodimerization between APJ and KOR and investigate their novel signal transduction characteristics. Co-immunoprecipitation (Co-IP), co-localization and bioluminescence resonance energy transfer (BRET) assays confirmed the heterodimerization of APJ and KOR. In APJ and KOR stably transfected HEK293 cells, treatment with APJ ligand apelin-13 or KOR ligand dynorphinA (1-13) resulted in higher phosphorylation levels of extracellular-regulated kinases 1/2 (ERK1/2) compared to HEK293 cells transfected with either APJ or KOR alone. The siRNA knockdown of either APJ or KOR receptor in human umbilical vein endothelial cells (HUVECs) resulted in significant reduction of the apelin-13 induced ERK activation. Additionally both forskolin (FSK)-induced cAMP levels and cAMP response element reporter activities were significantly reduced, whereas the serum response element luciferase (SRE-luc) reporter activity was significantly upregulated. Moreover, the ERK phosphorylation and SRE-luc activity were abrogated by the protein kinase C (PKC) inhibitor. These results demonstrate for the first time that human APJ forms a heterodimer with KOR and leads to increased PKC and decreased protein kinase A activity leading to a significant increase in cell proliferation, which may translate to the regulation of diverse biological actions and offers the potential for the development of more selective and tissue specific drug therapies.

1. Introduction

G protein-coupled receptors (GPCRs), the main targets for pharmacological agents, are now known to function in cells as homo or heterodimers or higher-order oligomers [1]. Dimerization may be involved in ligand binding, cell signaling, desensitization and receptor trafficking to increase the diversity of GPCR phenotypes [2-5]. As GPCRs are the most targeted group of proteins for the development of small molecule based therapeutic drugs, recent investigations have revealed that heterodimerization between co-expressed G protein-coupled receptors may be a common process and offers the potential for the development of more selective and tissue specific drugs [1,6].

The GPCR, APJ was first isolated from the human genomic DNA [7]. The APJ ligand apelin peptide was initially isolated from bovine stomach extracts [8]. Apelin is derived from a 77-amino-acid prepropeptide which is processed to several active molecular forms, such as apelin-13, apelin-17 and apelin-36 in different tissues [9]. While each of these isoforms has biological activity, the predominant isoform demonstrated to regulate the cardiovascular function appears to be [Pyr^1] apelin-13 [10]. Since its discovery, apelins has been shown to be involved in the regulation of cardiovascular functions, neuroprotection, pain and fluid homeostasis [10-12].

The opioid receptors are members of the family A of GPCRs, and include subtypes μ, δ, and κ (MOR, DOR, and KOR). It plays pervasive roles in pain perception, addiction, cardiovascular regulation, and neuroprotection [13-16].

Previous studies demonstrated that APJ and KOR share a number of common characteristics; they belong to class A subfamily of GPCRs superfamily and transduce signals through activation of Gi/o proteins

Abbreviations: APJ, apelin receptor; Bis II, Bisindolylmaleimide II [2-(1-[2-(1-Methyl-pyrro-lidino) ethyl]-1H-indol-3-yl)-3-(1H-indol-3-yl)-maleimide; BRET, bioluminescence resonance energy transfer; cAMP, cyclic AMP; Co-IP, co-immunoprecipitation; CRE, cAMP-response element; DynA(1-13), dynorphinA(1-13); ERK1/2, extracellular-regulated kinases 1/2; FSK, forskolin; IBMX, 3-isobutyl-1-methylxanthine; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; Human umbilical vein endothelial cells, HUVECs; IP, immunoprecipitate; KOR, kappa opioid receptor; Luc, luciferase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase A; SRE, serum response element.

* Corresponding author at: Department of Neurobiology, Jining Medical University, No.16 Hehua Road, Jining, Shandong, 272067, China. Tel.: +86 537 361 6002; fax: +86 5373616777.
E-mail addresses: tsmclyl@126.com (Y. Li), bbai@mail.jnmc.edu.cn (B. Bai).
[1] Present address: Department of Neurobiology, Taishan Medical University, No. 2 Yingsheng East Road, Taian, Shandong, China 271000. Tel.: +86 538 622 2151; fax: +86 538 622 2036.

0898-6568/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2011.12.012

that lead to the activation of extracellular-regulated kinases 1/2 (ERK1/2) [17-19] and inhibition of adenylyl cyclase activity [19,20]. They share similar distribution in the cardiovascular and central nervous system. Furthermore, they exhibited similar function in cardiovascular and neuroprotection function and pain regulation. We aim to study if these two types of GPCRs can dimerize. Furthermore, we explored whether the signal transduction of the heterodimer would be altered by apelin-13 or DynorphinA (1-13)[DynA(1-13)].

Bioluminescence resonance energy transfer (BRET) has become extremely popular for studying protein–protein interactions in living cells and real time [21]. Of particular interest is the ability to monitor dimerization of GPCRs, which have revealed that GPCRs often exist as homo- or heterodimers [21-26]. The BRET is based on fusing the Renilla luciferase (Rluc, energy donor) and enhanced green fluorescent protein (EGFP, energy acceptor) to either of two interacting proteins. Rluc oxidizes its substrate Coelenterazine h, resulting in the emission of energy. If a suitable energy acceptor is in close proximity (less than 100 Å), and is favorably oriented, this energy can be transferred. The excited acceptor molecule then emits energy at a longer wavelength [21,27]. In this study, BRET assays was used in detecting heterodimerization between APJ and KOR.

Here we provide the evidence for the first time that heterodimerization of human APJ and KOR occurs and this heterodimerization leads to novel signal transduction properties.

2. Materials and methods

2.1. Plasmid constructs

The pcDNA3.1-APJ plasmid was constructed previously [19]. The pcDNA3.1-KOR plasmid was obtained from UMR cDNA Resource Center (University of Missouri-Rolla). To construct HA-tagged apelin receptor (HA-APJ), Myc-tagged kappa opioid receptor (Myc-KOR), the incorporation of sequences encoding the hemagglutinin (HA) epitope tag (YPYDVPDYA) and the Myc epitope tag (EQKLISEEDL) into human APJ or KOR genes, respectively, was performed by PCR. The purified full-length cDNA of HA-APJ and Myc-KOR was digested and cloned into pcDNA3.1 plasmid. Rluc-hKOR was made using the forward primer 5'-ATAAGAATGCGGCCGCATGGAATCCCCGATTCAGATC-3' and reverse primer5'-CTCTAGACTCGAGTCATACTGG -3'and cloned into pRluc-N1 (BioSignal Packard); EGFP-hAPJ was made using 5'-CCGGAATTCATGGAGGAAGGTGGTGATTTTG -3' forward primer and 5'-CCGGGATCCGCTAGTCAACCACAAGGGTCTC -3' reverse primer and cloned into the pEGFP-C1 (Clontech). To study whether kappa opioid receptor specifically forms homo-dimer or nonspecifically heterodimerized with other G protein-coupled receptors, we also labeled human kappa opioid receptor and muscarinic M2 receptor with GFP using PCR methods. The resulting construct was confirmed by commercial sequencing.

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in culture medium containing high-glucose Dulbecco's modified Eagle's medium (DMEM) supplement with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C incubator in 5% CO_2. HEK293 cells stably expressing human APJ (HEK293-APJ cells) was described previously [19]. For the generation of cell lines stably expressing human KOR (HEK293-KOR cells), HEK293 cells were transfected with the pcDNA3.1-KOR plasmid using Lipofectamine 2000 (Invitrogen, Grand Island, USA) according to the manufacturer's instructions, transfected cells were cultured in DMEM in the presence of G418 (0.5 mg/ml) (Gibco, Invitrogen, Paisley, UK). Those that survived were subcultured in the selective process for eight weeks. Individual cell lines were cloned by limiting dilution. KOR expression was assessed by Western blot. For generation of cell lines stably co-expression human APJ and KOR (HEK293-APJ/KOR cells), HEK293 cells were co-transfected with pcDNA3.1-APJ and pcDNA3.1-KOR plasmids according to the previous described methods [19]. For some experiments, transient transfection was performed as previously described [19].

2.3. Immunostaining and confocal microscopy

HEK293 cells were grown on glass coverslips pretreated with a 0.1 mg/ml solution of poly-D-lysine in 6-well plates. Forty-eight hours after transfection with HA-APJ or Myc-KOR or both, cells were fixed with 4% paraformaldehyde in PBS and nonspecific binding was reduced by incubating cells with 3% bovine serum albumin in PBS-Triton X-100 (0.01%) for 1 h at room temperature. Cells were washed three times for 5 min with PBS-Triton X-100 (0.01%) then incubated with specific primary antibody (monoclonal Myc-antibody conjugated with Alexa-Fluor®594 (red) and anti-HA antibody) (Cell Signaling Technology, Danvers, USA) overnight at 4 °C. The cells were then washed with PBS-Triton X-100 as before and incubated with Alexa Fluor 488 (green) conjugated secondary antibodies (1:400) (Molecular Probes, Invitrogen) for 1 h at room temperature. Coverslips were washed with PBS and mounted in Vectashield Mounting Medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Peterborough, UK) on microscope slides. Cells were examined under an oil immersion objective (×63) using a Leica model DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK).

2.4. Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation were performed as described previously and a little modified [13,28], HEK293 cells in 60-mm dishes were cotransfected with HA-APJ and Myc-KOR or the vector control. Forty-eight hours later, the cells starved overnight followed by stimulation with 100 nM apelin-13 for 15 min and lysed in 800 μl cell lysis buffer (120 mM NaCl, 0.5% NP-40, 100 mM NaF, 50 mM Tris–Cl, pH 8.0). After clarification by centrifugation at 4 °C for 15 min at 161,000 g, 500 μl whole cell lysates were incubated with 2 μg of anti-HA antibody and 30 μl protein G-Sepharose beads for 4 h with gentle rotation at 4 °C. The beads were washed 4 times with the cell lysis buffer and precipitates were eluted with 2×SDS-PAGE sample buffer and analyzed by Western blot for anti-Myc and anti-HA immunoreactivity described above. To monitor the protein expression level, 10% of total cell lysates of each sample were used for Western blots.

2.5. BRET experiment

HEK293 cells were transiently cotransfected with vectors encoding Rluc fusion or EGFP fusion proteins. Twenty-four hours after transfection, cells were detached with 0.05% trypsin-0.53 mM EDTA, and cells at a density of 2 to 5×10^4 cells/well were distributed in a 96-well microplate (Corning 3600, white opaque plates). After another 24 h, cells were washed twice in PBS and resuspended in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose. Coelenterazine h substrate was added at a final concentration of 5 μM in the total volume of 50 μl/well. When appropriate, cells were pretreated with apelin-13 or DynA(1-13) at 100 nM final concentration as indicated for 15 min prior to addition of substrate. Readings were then immediately performed at 37 °C and at 0.5 s intervals and during several minutes using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) that allows the sequential integration of light signals detected with two filter settings (Rluc filter, 475±30 nm; and EGFP filter, 535±30 nm). The values were corrected by subtracting the background BRET signals detected when Rluc-hKOR was expressed alone. The BRET signal is reported as mBRET ($10^3\times$ BRET ratio). The calculated BRET signals were plotted as a function of the total fluorescence/luminescence ratios.