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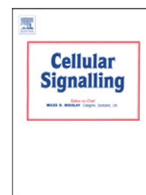

Heterodimerization of human apelin and bradykinin 1 receptors: Novel signal transduction characteristics

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ABSTRACT

Apelin receptor (APJ) and bradykinin 1 receptor (B1R) are involved in a variety of important physiological processes, which share many similar characteristics in distribution and functions in the cardiovascular system. This study explored the possibility of heterodimerization between APJ and B1R, and investigated the impact of heterodimer on the signal transduction characteristics and the physiological functions in human endothelial cells after stimulation with their agonists. We first identified the endogenous expression of APJ and B1R in HUVECs and their co-localization on HEK293 membrane. The constitutive heterodimerization between the APJ and B1R was then demonstrated by BRET and FRET assays. Stimulation with Apelin-13 and des -Arg^9-BK enhanced the phosphorylation of eNOS in HUVECs, which could be dampened by the knockdown of APJ or B1R, indicating the co-existence of APJ and B1R is critical for eNOS phosphorylation in HUVECs. Furthermore, APJ/B1R heterodimers were found to enhance the activity of PKC signaling pathway and increase intracellular Ca^{2+} concentration in HEK293 cells, which might be the mechanism of APJ/B1R heterodimers promoting the phosphorylation of eNOS and leads to increased Gαq, PKC signal pathway activities and a significant increase in cell proliferation. The results provide a new theoretical and experimental base for revealed intracellular molecular mechanisms of physiological function involved in the APJ and B1R and provide potential new targets for the development of drugs and treating cardiovascular disease.

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1. Introduction

G protein coupled receptors (GPCRs) are a link of signal transduction transferring extracellular stimuli to intracellular signals, widely involved in reproduction, development, growth, and many other physiological and pathological processes. It is thus the target for 40%–50% of all drugs on the market [1].

In recent years, a large number of studies have shown that GPCRs exert important roles in the form of heterodimers which further represent new features, such as agonist recognition, ligand binding, tissue specificity, G protein binding, signaling and trafficking change, and so on [2–4]. It has been found that the GPCR heterodimer is involved in many physiological processes. For example, the heterodimers of cannabinoid receptor and adenosine Alpha-2 a receptor may regulate the effect of movement mediated by cannabinoid in the striatum [5]. The interaction between μ-opioid receptors and CB1 cannabinoid receptors affects the physical process of synapse formation [6]. Besides, heterodimeric β1AR–β2AR exhibits altered pharmacological and signaling properties, resulting in more potent cAMP and contractile responses to agonist stimulation, while silencing ligand-independent spontaneous β2AR activity [7]. The capacity of GPCR dimerization to alter the G protein-coupling selectivity compared with those of the corresponding monomers has also been a central element in defining specific roles for the dopamine D1–D2 receptor heterodimers [8]. This pairing has been indicated to form a specific heterodimer in parts of the striatum [9] and to mediate a group of distinct signals through the engagement of $Ca^{2\pm}$ mediated signaling rather than the regulation of cAMP levels most normally anticipated to result from dopamine either D1 or D2 receptor activation. Therefore, it is of important significance to research GPCR heterodimerization which is beneficial to further elaborate the molecular mechanism of signal transduction, and discovery of new drug targets.

The apelin family is an endogenous active peptide and can activate G protein coupled receptor APJ. Apelin is widely distributed in the lung, heart, kidney, blood vessels, brain, and represents a wide range of biological effects [10–13]. Apelin-13, as the most representative peptide of the apelin family, plays important roles in lowering blood pressure and enhancing cardiac contraction in cardiovascular regulation.

Bradykinin (BK) is a vascular peptide generated from prekallikrein activated by kallikrein. It participates in regulating blood pressure,

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central pain, inflammation, and many other physiological and pathological processes. There are two bradykinin receptors: the B1 receptor (B1R) and the B2 receptor (B2R), which both belong to the G protein coupled receptor family [14,15]. BK is the specific agonist of B2R. des-Arg^9-BK and des-Arg^{10}-KD, generated by the Arg residues at the C-terminus cleaved from BK are the specific agonists of B1R.

It has been found that there are many overlapped functions and signal pathways in two systems of Apelin/APJ and BK/B1R. For example, they are both distributed in the cardiovascular system, and are involved in the cardiovascular homeostasis and the protection of the nervous system [16,17]. Both apelin-13 and des-Arg^9-BK are catalytic substrates for the Angiotensin Converting Enzyme II (ACE2) in the renin angiotensin aldosterone system (RAS) [18]. In the signal transduction aspect, they can bind to $G\alpha_i$ and $G\alpha_q$, regulate PKA and PKC signal pathways and are involved in cell proliferation, differentiation, morphology, cell skeleton construction and cell apoptosis [11,15]. Therefore, it is speculated that it is likely that APJ and B1R heterodimers exist in cells. Furthermore, the heterodimers between the APJ and B1R might have some effects on signal transduction and physiological pathology.

In the past twenty years, a series of techniques and methods to detect the interaction of proteins have been developed, such as classic immune co-precipitation, and the laser confocal. However, these methods have some limitations. On the one hand, they could change the natural state of the protein–protein interaction based on the mechanical and high centrifugal force as well as on the detergent cell lysis. On the other hand, they cannot provide time and space information of protein interaction in living cells. In recent years, resonance energy transfer technology has been widely applied, which overcomes the above shortcomings and can detect real-time and dynamic protein interactions in living cells [19–21]. In our present study, we pay attention to research the possibility of heterodimers between the APJ and B1R using resonance energy transfer technology. Meanwhile, we study the impact of these heterodimers on signal transduction and physiological functions, to further reveal the molecular mechanism of heterodimers in the cardiovascular system, and provide experimental basis for exploring new breakthroughs and new drug of hypertension and heart failure.

2. Materials and methods

2.1. Plasmid constructs

The pcDNA3.1-APJ and pcDNA3.1-B1R plasmids were obtained from UMR cDNA Resource Center (University of Missouri-Rolla). To construct pRluc-APJ, Human APJ was amplified using the sense primer: 5′-CGCG GATCCATGGAGGAAGGTGGTGAT TTTGAC-3′, and antisense primer: 5′-CCGGAATTCGTCAACCACAAGGGTCTCCTG GC-3′, and then the product was digested and cloned into pRluc-N1 (BioSignal Packard). To construct pEGFP-B1R and pEYFP-B1R, Human B1R was amplified using 5′-CCGCTCGAGATGGCATCATCCTGGCCCCCTCT-3′ as forward primer and 5′-CGCGGATCCATTCCGCCAGAAAAGTTGGAAGA-3′as reverse primer. The amplified fragments were digested and cloned into the pEGFP-N1 (Clontech) or pEYFP-N1 (Clontech), respectively. pECFP-APJ was amplified using forward primer 5′-CCC AAGCTTATGGAGGAAGGTGGTGATTTT GAC-3′ and reverse primer 5′-CCGGAATTCGTCAACCACAAGGGTCTCCTG GC-3′ and cloned into the pECFP-N1 (Clontech). The recombinant constructs were confirmed by sequencing.

pEYFP-APJ and pEGFP-APJ were constructed previously [10,22]. The plasmids, pSRE-Luc and pCRE-Luc, were purchased from Stratagene (La Jolla, Cal, USA). The plasmids pNFAT-RE-Luc and pRL-TK were purchased from Promega (Madison, WI, USA).

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured in MEM (Hyclone, China) with 10% heat inactivated fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. Human umbilical vein endothelial cells (HUVECs) were cultured in a M199 medium (pH 7.4) with 10% fetal calf serum, 30 μg/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. To generate cell lines stably expressing the APJ (HEK293-APJ cells), B1R (HEK293-B1R cells), and APJ/BB1R (HEK293-APJ/B1R cells), HEK293 cells were transfected with plasmid pcDNA3.1-APJ and pcDNA3.1-B1R respectively or together, using the Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions.

2.3. Reverse transcription-polymerase chain reaction

To detect the endogenous expression of APJ and B1R, total RNA was purified from HUVECs for reverse transcription-polymerase chain reaction (RT-PCR). The primers used for PCR amplification are: forward primer and reverse primer of B1R 5′-GAACATCACCGCCTGCATCC-3′ and 5′-AAGGCAACCACGAGCGTGAG-3′ (Product length: 225 bp); forward primer and reverse primer of APJ: 5′-TCAGCAGCTACCTCATCT TC-3′ and 5′-ACTGCACCTTAGTGGTGTTC-3′ (Product length: 231 bp). Ten microliters of the reaction mixture(s) were subsequently electrophoresed on a 1% agarose gel and visualized by ethidium bromide, using a 1-kb DNA ladder to estimate the band sizes. The PCR products from samples were purified from the 1% agarose gel using the QIAquick gel extraction kit (QIAGEN). PCR products were then sequenced in an automated DNA sequence, and the sequence data were analyzed using the Blast nucleic acid database searches (National Centre for Biotechnology Information, Bethesda, MD), confirming the identity of our products.

2.4. Immunostaining and confocal microscopy

HEK293 cells were grown on glass cover slips pretreated with a 0.1 mg/ml solution of poly-D-lysine in 6-well plates. Forty-eight hours after co-transfection with N-terminal HA-tagged APJ and C-terminal EGFP-tagged B1R, cells were fixed with 4% paraformaldehyde in PBS and nonspecific binding was reduced by incubating cells with 3% bovine serum albumin in the PBS-Triton X-100 (0.01%) for 1 h at room temperature. Cells were washed three times for five minutes with the PBS-Triton X-100 (0.01%) then incubated with Anti-HA antibody overnight at 4 °C. Cells were then washed with the PBS-Triton X-100 as before and incubated with Alexa-Fluor®594 (red) conjugated secondary antibodies (1:400) (Molecular Probes, Invitrogen) for 1 h at room temperature. Coverslips were washed with PBS and mounted in a Vectashield Mounting Medium with 4′, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Peterborough, UK) on microscope slides. Cells were scanned with a Leica model DMRE laser scanning confocal microscope (Leica, Milton Keynes, UK) as described previously [22]. For detecting GFP, fluorescein isothiocyanate fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected for 510–520 nm for GFP.

2.5. BRET experiment

HEK293 cells were transiently co-transfected with vectors encoding Rluc fusion or EGFP fusion receptors. Twenty-four hours after transfection, cells were detached with 0.05% trypsin-0.53 mM EDTA, and cells at a density of 2×10^5 cells per 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 a total volume of 50 μl/well. When appropriate, cells were pretreated with apelin-13 or des-Arg^9-BK at 100 nM or 1 nM final concentration as indicated for 15 min prior to addition of substrate. BRET experiment was detected previously [23]. Readings were then immediately performed at 37 °C and at 1-s intervals and during several minutes using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) which allowed the sequential integration of light signals detected with two filter settings (Rluc filter,430 $\pm$ 30 nm; and

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