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Adenosine A_1 receptors heterodimerize with β_1- and β_2-adrenergic receptors creating novel receptor complexes with altered G protein coupling and signaling

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ABSTRACT

G protein coupled receptors play crucial roles in mediating cellular responses to external stimuli, and increasing evidence suggests that they function as multiple units comprising homo/heterodimers and hetero-oligomers. Adenosine and β-adrenergic receptors are co-expressed in numerous tissues and mediate important cellular responses to the autocoid adenosine and sympathetic stimulation, respectively. The present study was undertaken to examine whether adenosine A_1ARs heterodimerize with β_1- and/or β_2-adrenergic receptors (β_1R and β_2R), and whether such interactions lead to functional consequences. Co-immunoprecipitation and co-localization studies with differentially epitope-tagged A_1, β_1, and β_2 receptors transiently co-expressed in HEK-293 cells indicate that A_1AR forms constitutive heterodimers with both β_1R and β_2R. This heterodimerization significantly influenced orthosteric ligand binding affinity of both β_1R and β_2R without altering ligand binding properties of A_1AR. Receptor-mediated ERK1/2 phosphorylation significantly increased in cells expressing A_1AR/β_1R and A_1AR/β_2R heteromers. β-Receptor-mediated cAMP production was not altered in A_1AR/β_1R expressing cells, but was significantly reduced in the A_1AR/β_2R cells. The inhibitory effect of the A_1AR on cAMP production was abrogated in both A_1AR/β_1R and A_1AR/β_2R expressing cells in response to the A_1AR agonist CCPA. Co-immunoprecipitation studies conducted with human heart tissue lysates indicate that endogenous A_1AR, β_1R, and β_2R also form heterodimers. Taken together, our data suggest that heterodimerization between A_1 and β receptors leads to altered receptor pharmacology, functional coupling, and intracellular signaling pathways. Unique and differential receptor cross-talk between these two important receptor families may offer the opportunity to fine-tune crucial signaling responses and development of more specific therapeutic interventions.

1. Introduction

G protein-coupled receptors (GPCR), which comprise the largest family of cell surface receptors, play crucial roles in regulating intracellular signaling. The conventional dogma is that GPCRs function as monomers at the cell surface and couple to G proteins in a one-to-one ratio. However, extensive research over the past decade has clearly established that GPCRs can function as multiple units ranging from dimers (homo and hetero) as well as higher order oligomers, even among members from distinct receptor families [1–3]. The functional consequences of GPCR heterodimerization include altered receptor pharmacology, signaling, trafficking, subcellular localization and receptor desensitization [1–3]. In the present study, we examined heterodimeric interactions between adenosine and β-adrenergic receptor families, which play important physiological roles in various tissues.

Adenosine and β-adrenergic receptors are co-expressed in numerous cell types including the mammalian heart. There are four distinct adenosine receptor (AR) subtypes (A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR), which exert differential effects in response to the autocoid adenosine. There are three mammalian β-adrenergic receptors subtypes — β_1R, β_2R, and β_3R. The β_1- and β_2R have distinct pharmacological and functional properties with the former being the primary mediator of the β-adrenergic-mediated increase in cardiac contractility [4,5]. Like most other GPCRs, adenosine and β-adrenergic receptors couple to specific G proteins with A_1AR coupling to inhibitory G protein ($G\alpha_i$) while β_1R and β_2R predominantly couple to stimulatory G protein ($G\alpha_s$). Signaling through their cognate G proteins, both adenosine and β-receptors stimulate various effector molecules including the mitogen

Abbreviations: GPCR, G protein-coupled receptors; A_1AR, A_1 adenosine receptor; β_1R & β_2R, β-adrenergic receptor subtypes; HEK-293, human embryonic kidney cells; IP, immunoprecipitation; IB, immunoblotting; IF, immunofluorescence; cAMP, cyclic adenosine monophosphate; pERK, phosphorylated extracellular signal-regulated kinase.

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activated protein kinase (MAPK) kinase pathway, leading to the activation of extracellular signal-regulated kinase (ERK).

There is significant evidence that A_1AR and both β_1 and β_2Rs function as homo- and heterodimers. It has been reported that the A_1AR heterodimerizes within the adenosine receptor family (A_{2a}AR), and also with D_1-dopamine and $mGlu_{1\alpha}$-glutamate receptors leading to various functional ramifications [6–8]. β_1- and β_2Rs have also been reported to form functional homodimers and heterodimers with receptors such α_{2A}-adrenergic, δ-opioid, and somatostain receptors [9–12]. The β_1R can heterodimerize with β_2Rs increasing β_2R internalization and signaling efficacy as well as switching signaling via $G\alpha_s$ to $G\alpha_i$ [13,14]. In addition, there is evidence for functional interaction between these two receptor families whereby A_1AR inhibits the contractile and biochemical responses of β-adrenergic receptor activation, referred to as the A_1AR anti-adrenergic effect [15–17]. However, the possibility of receptor heterodimerization between these two receptor types has not been evaluated previously.

Given that adenosine and β-adrenergic receptors are co-expressed in numerous cell types, and that A_1AR antagonizes the β-adrenergic receptor effects in the heart, we investigated whether the A_1AR can form functional heterodimers with β_1 and β_2Rs. In this study, the formation of heterodimers was assessed using co-immunoprecipitation and co-localization approaches with co-transfected HEK-293 cells and normal adult human heart tissue lysates. The functional impact of co-expression was assessed by measuring binding affinity of orthosteric radioligands as well as by examining agonist-induced activation of intracellular signaling pathways (cAMP production and ERK phosphorylation).

2. Materials and methods

2.1. Materials

Receptor agonists, antagonists, and other inhibitors were purchased from Tocris Bioscience (Ellisville, MO). All cell culture reagents and reagents for immunofluorescence were from Invitrogen (Carlsbad, CA). The Tropix cAMP Screen System was obtained from Applied Biosystems (Carlsbad, CA). Full length, N-terminally FLAG-tagged human β_1-adrenergic receptor in pcDNA3.1 + vector was a generous gift from Dr. Suleiman Bahouth (Department of Pharmacology, University of Tennessee Health Sciences Center). Full length, N-terminal hemagglutinin (HA) epitope-tagged human A_1AR, β_2-adrenergic receptor, adenosine A_3AR, and M_2 muscarinic receptor constructs in pcDNA3.1 + vector were purchased from Missouri S&T cDNA Resource Center (www.cdna.org). In order to have differential epitope tags, the HA-tag present on the A_1AR construct was replaced by inserting a Myc epitope tag (EQKLISEEDL) by polymerase chain reaction (PCR). High quality plasmid DNA was isolated using plasmid isolation kits available from QIAGEN (Valencia, CA). Epitope tag antibodies include THE anti-HA from GenScript Inc. (Piscataway, NJ), anti-FLAG-M2 and anti-Myc from Sigma-Aldrich (St. Louis, MO), HRP-conjugated antibodies anti-HA rat monoclonal-HRP antibody (Roche Applied Science, Indianapolis, IN), anti-FLAG-M2-HRP antibody, and anti-Myc-HRP antibody (Sigma-Aldrich, St. Louis, MO). Phosphorylated ERK (pERK 42/44) and HRP-conjugated GAPDH antibodies were obtained from Cell Signaling (Danvers, MA) and Sigma-Aldrich (St. Louis, MO), respectively. Protein G Dynabeads and Novex Tris-Glycine polyacrylamide gels were purchased from Invitrogen (Carlsbad, CA). Normal adult human heart tissue lysate was purchased from Novus Biologicals (Littleton, CO).

2.2. Cell culture and transfection

Human embryonic kidney (HEK-293) cells were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 1% penicillin/streptomycin in a 37 °C/5% CO_2/humidified incubator. Cells were transfected using a standard calcium phosphate method with 2 μg of DNA per receptor and harvested 48 h post-transfection for various assays, as described below. In single receptor transfections, transfected plasmid DNA levels were kept constant by using empty vector DNA (pcDNA 3.1 +).

2.3. Co-immunoprecipitation and Western blotting

Forty eight hours following transfection, cells were harvested and lysed in a modified RIPA buffer (1% Triton X-100, 50 mM Tris-pH 7.5, 150 mM NaCl, 5 mM EDTA, 100 mM iodoacetamide, and protease inhibitors). Solubilized lysates were incubated overnight in the presence of appropriate epitope tag antibodies. The antibody-bound protein complexes were pulled down with Protein G-Dynabeads and eluted with Laemmli sample buffer (without DTT or β-mercaptoethanol). Samples were resolved by denaturing PAGE and protein complexes were visualized by Western blotting using primary antibodies conjugated to HRP: anti-HA rat monoclonal-HRP antibody, anti-FLAG-HRP antibody, and anti-Myc-HRP antibody. For human heart lysates, 200 μg of total protein was solubilized in RIPA buffer and incubated with antibodies against A_1AR and βR overnight followed by immunoprecipitation as described above. Total heart lysates (25 μg) and A_1AR-antibody bound complexes were detected by immunoblotting using antibodies against β_1- and β_2R. Converse immunoprecipitation reactions were carried out using anti-βR antibodies and visualized with anti-A_1AR antibody.

2.4. Co-localization via immunofluorescence microscopy

HEK-293 cells were plated on poly-D-lysine coated coverslips and transfected singly with A_1AR, β_1- and β_2-adrenergic receptors or co-transfected with A_1AR/β_1- and A_1AR/β_2-adrenergic receptors. Forty-eight hours post-transfection, cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde. Cells were then used without permeabilization or with permeabilization (0.2% Triton-X 100), blocked with normal goat serum, and incubated with various primary antibodies in combination: rat monoclonal anti-HA, mouse monoclonal anti-FLAG-M2, and rabbit polyclonal anti-cMyc. Cells were washed and incubated with AlexaFluor-conjugated secondary antibodies: AF-488, AF-594, or AF-350, as indicated in the figures. Cover slips were mounted with ProLong Gold antifade reagent and visualized with a Leica SD6000 confocal microscope.

2.5. Receptor-mediated activation of ERK

Cells grown in 6-well cell culture plates were transiently transfected in various combinations as described above. Forty-four hours post-transfection, cells were starved in serum-free DMEM for 4 h in the presence of adenosine deaminase (2 U/mL). Cells were then treated for 5 min with the A_1AR agonist CCPA (250 nM) or βR agonist isoproterenol (ISO, 500 nM) and lysed with RIPA buffer containing protease and phosphatase inhibitor cocktails. Receptor-mediated activation of the mitogen-activated protein kinase (MAPK) pathway was assessed by Western blotting using anti-phospho extracellular regulated kinase (pERK 42/44) antibody. The pERK signal was normalized to total protein present in the samples by probing with an HRP-conjugated anti-GAPDH antibody. All drug treated samples were also normalized against vehicle-treated samples to determine basal activation of pERK. Densitometric quantification was carried out using the NIH Image J software and groups compared using two-tailed unpaired *t*-test.

2.6. cAMP assay

Following serum starvation in the presence of adenosine deaminase as described above, cells were treated for 20 min with the phosphodiesterase inhibitor rolipram (50 μM) prior to 10 min exposure to ISO or CCPA. Cells were then lysed and intracellular cAMP levels were determined by a competitive immunoassay kit, Tropix cAMP Screen