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Adenosine A₁ receptors heterodimerize with β_1 - and β_2 -adrenergic receptors creating novel receptor complexes with altered G protein coupling and signaling

P. Charukeshi Chandrasekera¹, Tina C. Wan², Elizabeth T. Gizewski², John A. Auchampach², and Robert D. Lasley¹

¹Department of Physiology, Cardiovascular Research Institute, School of Medicine, Wayne State University, Detroit, MI

²Department of Pharmacology and the Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, WI

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 autocrine adenosine and sympathetic stimulation, respectively. The present study was undertaken to examine whether adenosine A₁ARs heterodimerize with β_1 - and/or β_2 -adrenergic receptors (β_1 R and β_2 R), and whether such interactions lead to functional consequences. Co-immunoprecipitation and co-localization studies with differentially epitope-tagged A₁, β_1 , and β_2 receptors transiently co-expressed in HEK-293 cells indicate that A₁AR forms constitutive heterodimers with both β_1 R and β_2 R. This heterodimerization significantly influenced orthosteric ligand binding affinity of both β_1 R and β_2 R without altering ligand binding properties of A₁AR. Receptor-mediated ERK1/2 phosphorylation significantly increased in cells expressing A₁AR/ β_1 R and A₁AR/ β_2 R heteromers. β -receptor-mediated cAMP production was not altered in A₁AR/ β_1 R expressing cells, but was significantly reduced in the A₁AR/ β_2 R cells. The inhibitory effect of the A₁AR on cAMP production was abrogated in both A₁AR/ β_1 R and A₁AR/ β_2 R expressing cells in response to the A₁AR agonist CCPA. Co-immunoprecipitation studies conducted with human heart tissue lysates indicate that endogenous A₁AR, β_1 R, and β_2 R also form heterodimers. Taken together, our data suggest that heterodimerization between A₁ 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.

Keywords

G protein coupled receptors; heterodimerization; adenosine receptor; β -adrenergic receptors

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To whom correspondence should be addressed: Robert D. Lasley, Department of Physiology, Wayne State University School of Medicine, 421 E. Canfield Ave, 1104 Elliman Bldg, Detroit, MI 48201, Tel: (313)-577-9444, Fax: (313)-577-8716, rlasley@med.wayne.edu.

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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_1 AR, A_{2A} AR, A_{2B} AR, and A_3 AR), which exert differential effects in response to the autocrine adenosine. There are three mammalian β -adrenergic receptors subtypes— β_1 R, β_2 R, and β_3 R. The β_1 - and β_2 R 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_1 AR coupling to inhibitory G protein (G_{α_i}) while β_1 R and β_2 R predominantly couple to stimulatory G protein (G_{α_s}). Signaling through their cognate G proteins, both adenosine and β -receptors stimulate various effector molecules including the mitogen activated protein kinase (MAPK) kinase pathway, leading to the activation of extracellular signal-regulated kinase (ERK).

There is significant evidence that A_1 AR and both β_1 and β_2 Rs function as homo- and heterodimers. It has been reported that the A_1 AR 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_2 Rs have also been reported to form functional homodimers and heterodimers with receptors such as α_{2A} -adrenergic, δ -opioid, and somatostatin receptors [9–12]. The β_1 R can heterodimerize with β_2 Rs increasing β_2 R internalization and signaling efficacy as well as switching signaling via G_{α_s} to G_{α_i} [13, 14]. In addition, there is evidence for functional interaction between these two receptor families whereby A_1 AR inhibits the contractile and biochemical responses of β -adrenergic receptor activation, referred to as the A_1 AR 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_1 AR antagonizes the β -adrenergic receptor effects in the heart, we investigated whether the A_1 AR can form functional heterodimers with β_1 and β_2 Rs. 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_1 AR, β_2 -adrenergic receptor, adenosine A_3 AR, 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_1 AR 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% CO₂/humidified incubator. Cells were transfected using a standard calcium phosphate method with 2 μ g of DNA per receptor and harvested 48 hours 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 μ g of total protein was solubilized in RIPA buffer and incubated with antibodies against A_1 AR and β R overnight followed by immunoprecipitation as described above. Total heart lysates (25 μ g) and A_1 AR-antibody bound complexes were detected by immunoblotting using antibodies against β_1 - and β_2 R. Converse immunoprecipitation reactions were carried out using anti- β R antibodies and visualized with anti- A_1 AR antibody.

2.4. Co-localization via immunofluorescence microscopy

HEK-293 cells were plated on poly-D-lysine coated coverslips and transfected singly with A₁AR, β_1 - and β_2 -adrenergic receptors or co-transfected with A₁AR/ β_1 - and A₁AR/ 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 hours in the presence of adenosine deaminase (2U/mL). Cells were then treated for 5 min with the A₁AR agonist CCPA (250 nM) or β R 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 μ M) 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 System, according to the manufacturer's instructions. All samples were normalized to the total protein amount, as determined by the method of Bradford. Vehicle treated samples were used to determine basal cAMP levels. cAMP levels normalized to the total protein concentration were further normalized against the maximal receptor stimulation obtained from single receptor transfections, as described in the figure legends.

2.7. Radioligand binding assay

Radioligand binding assays were conducted with membranes prepared from HEK 293 cells transiently expressing the various combinations of adenosine and β -adrenergic receptors using the agonist radioligand *N*⁶-(4-amino-3-[¹²⁵I]iodobenzyl) adenosine-5'-*N*-methyl-carboxamide ([¹²⁵I]I-AB-MECA) in assays with the A₁R and the antagonist radioligand [¹²⁵I]iodo-(-)-cyanopindolol (¹²⁵I-CYP) in assays with β_1 or β_2 Rs. [¹²⁵I]I-AB-MECA and ¹²⁵I-CYP were prepared by radioiodination of the precursors AB-MECA and CYP, respectively, using the chloramine-T method [18]. Receptor transfected HEK-293 cell membranes were prepared as described previously [18]. For saturation equilibrium radioligand binding studies, cell membranes (2 μ g protein/tube for β receptor assays and 50 μ g/tube for A₁R assays) were tested against a range of 6–8 increasing concentrations of either radioligand. After incubating at room temperature for 3 h, the membranes were collected by filtration over Whatman GF/C filters and the radioactivity trapped in the filters was quantified using a gamma counter. Non-specific binding was determined in the presence

of alprenolol (10 μ M for β receptors) or adenosine-5'-*N*-ethylcarboxamide (100 μ M for A_1 Rs). In all assays, specific binding of the radioligands fit optimally to a single-site binding model: $y = (B_{\max} \times [L]) / (K_d + [L])$, from which $B_{\max}$ and K_d values were obtained.

3. RESULTS

3.1. A_1 ARs co-immunoprecipitate with both β_1 and β_2 receptors

The ability of the A_1 AR to heterodimerize with β_1 or β_2 Rs was first assessed using a co-immunoprecipitation approach in HEK-293 cells. Using appropriate antibody pairs, each of the receptors was immunoprecipitated and the presence of additional receptor subtypes in the precipitates was assessed by Western blotting. In order to show that heterodimerization only occurs in co-transfected cells, cells singly transfected with each receptor were first mixed and then lysed. As shown in Figure 1a, both β_1 and β_2 Rs co-immunoprecipitated with the A_1 AR in lysates prepared from cells co-expressing A_1 AR/ β_1 R and A_1 AR/ β_2 R, but not in lysates prepared from a mixture of cells expressing each of the receptors alone. The doublet present in immunoblots for the A_1 AR likely represents the glycosylated (higher band) and unglycosylated (lower band) forms of the receptor, as reported previously [19].

In order to ensure specificity of interaction between A_1 AR and β Rs, co-immunoprecipitation experiments were conducted in which A_1 AR was substituted with the only other G_i -coupled adenosine receptor subtype, the A_3 AR, and a G_i -coupled receptor from a completely different family, muscarinic M_2 receptor (M_2 MR). HEK-293 cells were co-transfected with A_3 AR/ β_1 R or M_2 MR/ β_1 R and co-immunoprecipitation was performed as described above. As shown in Figure 1b, heterodimerization was not detectable with either A_3 AR/ β_1 R or M_2 MR/ β_1 R. These immunoprecipitates were also probed with the respective epitope tag antibody for each receptor type to ensure that receptors were properly expressed (1b, right panel). Taken together, these results suggest that the A_1 AR is capable of forming specific stable heterodimeric complexes with either β_1 or β_2 Rs.

3.2 A_1 ARs co-localize with β_1 and β_2 receptors at the cell plasma membrane

The potential for co-localization of A_1 ARs and β receptors in HEK-293 cells was assessed using immunofluorescence microscopy. As shown in Figure 2, confocal images of HEK-293 cells co-expressing A_1 ARs and either β receptor subtype revealed a similar cellular expression pattern for the A_1 AR and both β receptor isoforms in co-transfected cells. In these non-permeabilized cells, it is clear that all three of the receptors were localized at the plasma membrane, and the merged images revealed almost complete overlap in the cellular expression pattern between the A_1 AR and β -receptors. These results indicate that A_1 AR and β R are capable of co-localizing at the plasma membrane, providing further support for their propensity for dimerization.

3.3 Ligand binding properties of A_1 ARs and β Rs during co-expression

Saturation radioligand binding was performed with membranes isolated from cells expressing various combinations of A_1 , β_1 , and β_2 receptors to determine whether co-expression influences orthosteric ligand binding affinity. As shown in Table 1, affinity of the A_1 AR for the agonist radioligand [125 I]I-AB-MECA did not change when co-expressed with either β_1 or β_2 receptors. In contrast, affinity of both the β_1 R and the β_2 R for the antagonist radioligand 125 I-CYP increased 1.6-fold and 3.7-fold when co-expressed with the A_1 AR. It is notable that the calculated $B_{\max}$ for both of the radioligands was significantly reduced by ~50% in assays using membranes from cells co-expressing receptors. These data suggest that dimerization may alter characteristics of the orthosteric binding site of β -receptors, but not the A_1 AR.

3.4 G protein-coupled signaling properties of A₁AR and βRs during co-expression

The functional consequences of co-expressing adenosine and β-adrenergic receptors were first evaluated at the level of MAPK activation, since both adenosine and β-adrenergic receptors increase ERK phosphorylation. As shown in Figure 3, when cells singly expressing A₁AR, β₁R and β₂R were treated with their selective agonists there was an increase in pERK1/2 compared to the basal levels in vehicle-treated cells. There were no differences among basal pERK1/2 levels or total ERK protein expression levels under all receptor transfection combinations (data not shown). As depicted in the representative western blot in Figure 3a and summarized in Figure 3c, the βR agonist isoproterenol (ISO, 500 nM) exerted similar effects on ERK phosphorylation in the β₁R singly and A₁AR/β₁R dually transfected systems. In contrast, β₂R receptor stimulation with ISO in A₁AR/β₂R cells was associated with significantly increased (2.29 ± 0.16 -fold) pERK1/2 levels compared to that of the β₂R singly transfected cells.

In the next set of experiments, the activation of ERK by A₁AR was tested using the A₁AR selective agonist CCPA (250 nM). When cells singly expressing A₁AR were treated with CCPA, there was a significant increase in ERK phosphorylation compared to vehicle, confirming previous observations that A₁AR can activate the MAPK pathway (Figure 3b). When A₁AR/β₁R dually transfected cells were treated with CCPA, there was a significant, yet relatively small increase in pERK of only 1.46 ± 0.14 -fold compared to the dramatic 9.61 ± 0.59 -fold increase observed in ERK phosphorylation in A₁AR/β₂R dually transfected cells (Figure 3c). Taken together, these data suggest that dimerization with A₁AR alters the ability of β₂R to promote MAPK activation but not that of β₁R, whereas activation of MAPK by A₁AR is significantly altered when dimerization occurs with both βR subtypes.

The effect of receptor co-expression on G_s protein-coupled activation of adenylyl cyclase and cAMP production was also examined. As expected, a significant increase in cAMP production was observed upon ISO (5 nM) stimulation in cells expressing β₁ or β₂Rs alone. Basal cAMP levels in these cells ranged from 4.8–6.1 pmol/mg protein and increased to 2000–3000 pmol/mg protein in response to ISO; data were normalized to maximal ISO response in cells singly transfected with each receptor subtype. As shown in Figure 4a, there was no significant increase in cAMP levels in response to ISO in cells dually expressing A₁AR and β₁R. However, the level of cAMP production in response to ISO was markedly less in cells co-expressing A₁AR and β₂Rs. In these cells, the cAMP concentration that was achieved following ISO stimulation was decreased by ~40% in comparison to the response seen in cells expressing the β₂R alone (Figure 4b). These data suggest that A₁AR/β₂R heterodimer formation appears to modulate G protein coupling leading to cAMP formation, but this appears not to be the case with the A₁AR/β₁R heterodimer.

The A₁AR couples to the inhibitory G-protein (G_i) to inhibit the production of cAMP, thus the effect of co-expressing this receptor with the β-receptors was assessed by determining the effects of the A₁ agonist CCPA on forskolin (FSK)-induced increases in cAMP production. There were no differences in basal cAMP levels in the different groups of transfected cells (range: 4.9–6.4 pmol/mg protein), nor were there differences in responses to FSK (10 μM), which increased cAMP to ~1200 pmol/mg protein in all groups. As shown in Figure 4c, when cells singly expressing the A₁AR were simultaneously treated with CCPA and FSK, cAMP production was significantly decreased in a concentration-dependent manner ($59 \pm 1\%$ of control when co-stimulated with 0.1 nM CCPA and $27 \pm 2\%$ of control when stimulated with 1 nM CCPA). However the inhibitory effect of CCPA (1 nM) was completely absent in cells co-expressing A₁ARs with either β₁ or β₂Rs. Therefore, it appears that heterodimer formation with either β receptor subtype may impede coupling of the A₁AR to inhibition of adenylyl cyclase and subsequent reduction in cAMP.

3.5. Endogenous A₁AR co-immunoprecipitates with endogenous β_1 and β_2 receptors

Since it was clear that heterologously expressed A₁AR heterodimerizes with β_1 R and β_2 R, the ability of endogenously expressed A₁AR to heterodimerize with β Rs was also assessed using a co-immunoprecipitation approach. Using normal adult human heart tissue lysates and appropriate antibody pairs, each of the receptors was co-immunoprecipitated and the presence of additional receptor subtypes in these immunoprecipitates was assessed by Western blotting. As shown in Figure 5, both β_1 and β_2 Rs co-immunoprecipitated with the A₁AR in human heart tissue lysates. These data suggest that endogenously expressed A₁AR is also capable of forming stable heterodimeric complexes with β_1 and β_2 Rs.

4. DISCUSSION

G protein-coupled receptors from distinct families are ubiquitously co-expressed in numerous tissue types and have been shown to interact with one another at various levels including receptor, G protein, and downstream signal transduction pathways [1–3]. There is extensive evidence that the A₁AR and both β_1 - and β_2 -adrenergic receptors can form heterodimers with multiple different GPCRs. The present findings describe the first report that the A₁AR can heterodimerize with members of the β -adrenergic receptor family. Using a heterologous transient transfection system with HEK-293 cells, our results suggest that co-expression of A₁ARs with β_1 - or β_2 -adrenergic receptors resulted in the formation of functional heterodimers with altered pharmacological and signaling properties. Furthermore, our data also suggest that these receptors form constitutive heterodimers in the human heart.

Our observations indicate that A₁AR can form heterodimers with β_1 - and β_2 -adrenergic receptors. Co-immunoprecipitation studies revealed that the A₁AR forms stable heteromeric complexes only when the different receptors were co-expressed in the same cell. The inability to co-immunoprecipitate such receptor complexes from mixtures of cells singly expressing these receptors indicate that the observed heterodimerization is not due to aggregation artifacts induced during detergent solubilization. In fact, preliminary data obtained with lysates prepared from different detergents (such as NP-40, β -maltoside, digitonin, C₁₂E₈, Brij-35, and CHAPS) yielded the same observations, confirming that these heteromers are not detergent-induced aggregates. Furthermore, our observations are not due to spurious disulfide bond formation since these complexes were stable in the presence of high concentrations of iodoacetamide during cell lysis and co-immunoprecipitation procedures. These dimers were detected under basal conditions in the absence of exogenous receptor ligands. Since all cells, including HEK-293 cells, release some adenosine into the extracellular space, as a byproduct of ATP catabolism, it is possible that dimer formation was dependent on the presence of endogenous adenosine which activates the A₁AR. However, preliminary studies indicated that the inclusion of adenosine deaminase (which catabolizes adenosine to inosine, which is not an agonist for the A₁AR) had no effect on co-immunoprecipitation patterns. Thus, the ability to co-immunoprecipitate heterodimers in the presence and absence of adenosine deaminase suggests that this dimerization is constitutive.

In order to further validate the specificity of interaction between A₁AR and the β R subtypes, co-immunoprecipitation was conducted using cells in which G_i-coupled A₁AR was substituted with the only other G_i-coupled AR, the adenosine A₃ receptor (A₃AR), or another G_i-coupled receptor from a completely different family, muscarinic M₂ receptor (M₂MR). M₂MR was chosen as a suitable candidate because its receptor attenuates the cardiac β -adrenergic contractile response in much the same way A₁AR does [20]. No heterodimerization was observed between A₃AR/ β_1 R or M₂MR/ β_1 R, and these results further confirm that the observed heterodimeric interactions are specific to A₁AR and the β R subtypes and not the result of possible overexpression or aggregation artifacts.

Double-label immunofluorescence data indicated that these receptors co-localize at the plasma membrane. Although co-localization does not confirm heterodimerization, assays designed to evaluate receptor-mediated cellular signaling confirmed that these complexes likely exist as dimeric cell surface receptors (see results below). For each receptor combination, radioligand binding data indicated that a 50% reduction in receptor density was observed when the receptors were co-expressed. It is possible that dimer formation may influence surface expression or the turnover rate of the receptors, but a more likely explanation is that these cells are operating at maximal capacity in this overexpression system, and cannot maintain expression of both receptor subtypes when they are co-transfected with two cDNA constructs. It has been widely reported that most GPCR dimerization occurs at the ER, during receptor biosynthesis. The decreased receptor expressions in the co-transfected cells did not seem to interfere with receptor activity since significant functional alterations (including increased functional activity) occurred in co-transfected cells even with lower receptor density compared to the singly transfected cells.

Formation of these heterodimers appeared to influence A₁AR signaling. A₁AR-mediated ERK activation was not significantly altered when it was co-expressed with β_1 Rs, but a prominent change occurred when it was expressed with the β_2 R where pERK levels increased 9-fold. Thus, these data suggest that dimer formation increased the efficacy of the A₁AR to couple to ERK signaling. In contrast, the ability of the A₁AR to inhibit forskolin-induced cAMP production was blocked in both A₁AR/ β_1 R and A₁AR/ β_2 R co-transfected cells. These differences do not appear to be due to altered A₁AR binding properties (Table 1), nor do they appear to be an artifact of co-transfection since the responses to forskolin were comparable in the single and co-transfection groups. Formation of GPCR heterodimers, including those with both A₁AR and β R subtypes, has been reported to alter normal G protein coupling exhibited by the monomeric form of the receptor [21–23]. It is possible that the inter- and intra-molecular interactions that occur in the formation of the A₁AR/ β_1 R and A₁AR/ β_2 R dimers hinder proper coupling of the A₁AR to G_i proteins, as has been suggested for other heterodimers [24–26]. Alternatively, it is possible that the A₁AR transactivates the β_1 R and β_2 R receptors in the heterodimers rendering the heterodimers G_s protein-coupled during A₁AR agonism. The lack of correlation between changes in MAPK activation and cAMP production may be explained by G protein independent MAPK activation mechanisms such as β -arrestin mediated MAPK activation, which has been observed with β_2 and A₁ARs [27, 28], and it is possible that this pathway may also be altered due to dimerization.

Co-expression of the A₁AR exerted subtype specific effects on β_1 R and β_2 R ligand binding affinities and function. The binding affinity (K_d) of the β_1 R was increased 1.6-fold when co-expressed with the A₁AR; this modest change in binding affinity was associated with only minor changes in ERK phosphorylation, and no change in the cAMP response. Thus, it appears that the β_1 R undergoes limited pharmacological and functional alterations when it dimerizes with the A₁AR. This could be due to the fact that β_1 R expression was ~ 7-fold greater than that achieved with the A₁AR in co-transfected cells. In contrast the β_2 R, which showed a similar level of expression as compared to the A₁AR, exhibited almost a 4-fold increase in agonist binding affinity when co-expressed with the A₁AR. The functional data indicate a 2-fold increase in ERK phosphorylation, but a ~40% reduction in the β_2 R-mediated cAMP production in response to ISO when the β_2 R was co-expressed with the A₁AR. It is possible that the β_2 R operates primarily via a G_s-coupled mechanism when expressed alone (consistent with large increases in cAMP) and switches to a G_i-coupled system when it constitutively dimerizes with the A₁AR (consistent with a decrease in cAMP production). As described above, GPCR heterodimerization can be associated with changes in preferences for specific G proteins, and there is evidence that the β_2 R may switch G protein coupling from G_s to G_i [28]. Furthermore, it is possible that the G_s-coupling

efficiency for the β_2 R is altered when it is co-expressed with A_1 AR due to changes that occur in the $\beta\gamma$ -subunit composition, which has been shown to contribute directly to the coupling specificity of β_2 R to G_s [30].

Our present observations may have physiological relevance for cell types in which these two receptor types are co-expressed, particularly the cardiovascular system. All three of these receptors are expressed in ventricular cardiomyocytes, where the A_1 AR is known to exert profound anti-adrenergic effects. Stimulation of the A_1 AR in these cells exerts essentially no direct effects on intracellular calcium, cAMP, or contractility, but blunts β R induced increases in these parameters [15–17]. Although it is generally thought that the A_1 AR anti-adrenergic effect is due to reductions in cAMP, previous reports indicated that high affinity agonist binding of the β R and G_s -protein cycling are significantly reduced in rat myocardial membranes treated with an A_1 AR agonist [31, 32]. Our findings suggest that A_1 AR dimerization with β_1 and/or β_2 Rs may play a role in the anti-adrenergic effect mediated by adenosine and A_1 AR agonists. In this regard, our co-immunoprecipitation data using human heart lysates suggest that endogenous β_1 - and β_2 R can form constitutive heterodimers with endogenous A_1 AR, implicating possible functional ramifications *in vivo* in endogenous tissue. Although there is no direct evidence to support this hypothesis in cardiac tissue, there are several reports suggesting that receptor heterodimerization does modulate β R-induced increases in contractility. There is evidence that β_1 R/ β_2 R heterodimerization in cardiac ventricular myocytes modulates β R-induced positive inotropy [5]. In addition, pharmacological blockade of the A_1 AR and gene deletion of the A_1 AR may both potentiate β R-induced increases in cardiac contractility [33, 34].

Given the importance of both adenosine and β -adrenergic receptor systems in the modulation of cardiac contractility and response to stress, receptor heterodimerization is likely to have significant functional ramifications, particularly under pathophysiological conditions in which receptor expression levels are altered. The level of expression and/or function of A_1 AR and β R receptor subtypes have been reported to be altered in heart failure [4, 35, 36]. Changes in activity even in only one of these receptor subtypes and the subsequent heteromeric interactions may alter the physiological effects of adenosine and sympathetic system activation. These studies may have significant potential therapeutic impact as well. It is estimated that ~40% of drugs on the market today target GPCRs, and the results of receptor heterodimerization studies, such as our present findings, suggest that targeting only one receptor system may have significant effects on another GPCR. The development of more selective drugs that can exert dual interactions with two different GPCRs that dimerize may prove to be more beneficial than those drugs that target only one receptor at a time. For example, the cardiac angiotensin II receptor and β_1 adrenergic receptor form functional heterodimers and a single antagonist can dually inhibit both receptor functions [37]. The development of agonists capable of modulating the properties of heterodimer units would contribute to more efficient and selective control of signaling cascades. Our observations may elucidate a novel signaling pathway that could potentially be targeted and manipulated for therapeutic use.

5. CONCLUSION

We report here for the first time that human A_1 AR forms functional heterodimers with human β_1 R and β_2 R where heterodimerization leads to altered receptor pharmacology, functional coupling, and receptor-mediated intracellular signaling. Unique and differential cross talk between such receptors may significantly modulate the function in tissues where these are co-expressed. Such novel and differential molecular interactions between important receptor families also offer the opportunity to fine-tune crucial signaling responses and development of more specific therapeutic interventions.

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ABBREVIATIONS

GPCR	G protein-coupled receptors
A₁AR	A ₁ adenosine receptor
β₁R & β₂R	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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HIGHLIGHTS

- Human adenosine A₁ receptors heterodimerize with β_1 and β_2 adrenergic receptors.
- This causes alterations in receptor pharmacology, coupling, and downstream signaling.
- A₁, β_1 , β_2 heterodimerization creates novel and differential molecular interactions.
- Such cross-talk leads to physiological consequences and represent new drug targets.

Figure 1.

Heterodimerization of A₁AR with β₁R and β₂R assessed by co-immunoprecipitation. (A) Epitope-tagged recombinant human A₁AR/β₁R and A₁AR/β₂R were transiently co-expressed in HEK-293 cells and the receptor heterodimers were captured and detected using co-immunoprecipitation and Western blotting, as described in Materials and Methods. Cell lysates (L) were immunoprecipitated (I) using antibody pairs as indicated. Control immunoprecipitations were also carried out using cell lysates from a mixture (M) of HEK-293 cells individually expressing each receptor type; (B) Epitope-tagged recombinant human A₃AR/β₁R and M₂MR/β₁R were co-transfected, co-immunoprecipitated, and probed with the epitope tag antibody pairs as shown. IP, immunoprecipitation; IB, immunoblot; MW, molecular weight. Data are representative of 2–3 independent experiments.

Figure 2. Heterodimerization of A_1 AR with β_1 R and β_2 R assessed by immunofluorescence co-localization. Cell surface co-localization was confirmed by double-label immunofluorescence studies conducted in fixed, non-permeabilized HEK-293 cells co-expressing epitope-tagged recombinant human HA- A_1 AR/FLAG- β_1 R and myc- A_1 AR/HA- β_2 R. Note that receptors remain co-localized at the cell surface as depicted by the overlay in purple (for A_1 AR/ β_1 R) or yellow (A_1 AR/ β_2 R). Data are representative of 2–3 independent experiments.

Figure 3.

Agonist-mediated activation of MAPK pathway – phosphorylation of ERK1/2. Transiently transfected HEK-293 cells individually expressing epitope-tagged recombinant A_1 AR, β_1 R, and β_2 R or cells co-expressing A_1 AR/ β_1 R and A_1 AR/ β_2 R receptors were stimulated with their respective agonists and the activation of MAPK was assessed by immunoblotting using an anti-phospho ERK1/2 antibody and quantified by densitometry. Representative immunoblots for each category are shown here: (A) cells individually expressing β_1 R and β_2 R or co-expressing A_1 AR/ β_1 R and A_1 AR/ β_2 R were stimulated with β R-agonist isoproterenol (ISO); (B) cells individually expressing A_1 AR or co-expressing A_1 AR/ β_1 R and A_1 AR/ β_2 R stimulated with A_1 AR agonist CCPA; (C) summary of all densitometry data representative of three independent experiments conducted in duplicate or triplicate (mean $\pm$ SEM). * $p < 0.05$, single receptor expression vs. co-expression.

Figure 4. Agonist-mediated generation of cAMP. HEK-293 cells individually expressing A_1 AR, β_1 R, and β_2 R or cells co-expressing A_1 AR/ β_1 R and A_1 AR/ β_2 R receptors were stimulated with their respective agonists and the intracellular cAMP levels were measured using a competitive cAMP ELISA kit. (A) β_1 R stimulated with 5 nM isoproterenol (ISO) and data are represented as % change from maximal stimulation resulting from single β_1 R expression. (B) β_2 R stimulated with 5 nM ISO and data are represented as % change from maximal stimulation resulting from single β_2 R expression. * P <0.05, β_2 R vs. A_1 AR/ β_2 R expression. (C) For A_1 AR, the agonist CCPA (‡ 0.1 nM CCPA and 1 nM CCPA for all other experiments) was used in conjunction with forskolin (FSK) to determine the concentration-dependent reduction of maximal FSK (10 μ M)-stimulated cAMP. Data are normalized to maximal cAMP response generated by FSK treatment in cells singly expressing A_1 AR. ‡ P <0.0001, A_1 AR (FSK only) vs. A_1 AR (FSK + 0.1 nM CCPA); * P <0.0001, A_1 AR (FSK only) vs. A_1 AR (FSK + 1 nM CCPA); # P <0.0001 for A_1 AR (FSK+1 nM CCPA) vs. A_1 AR/ β_1 R and A_1 AR/ β_2 R (FSK + 1 nM CCPA). For all cAMP experiments, data shown represent average results (mean $\pm$ SEM) from 3 independent experiments performed in duplicate or triplicate.

Figure 5. Heterodimerization of endogenously expressed A₁AR with β₁R and β₂R Co-immunoprecipitation of endogenous A₁AR, β₁R, and β₂R was conducted with normal adult human heart tissue lysate using antibodies specific to each receptor. Receptor heterodimers were captured and detected as described in Materials and Methods. L, lysate; I, immunoprecipitate; IP, immunoprecipitation; IB, immunoblot; MW, molecular weight.

Table 1

Equilibrium, saturation radioligand binding data

	¹²⁵ I]-AB-MECA binding		¹²⁵ I]-CYP binding	
	K _d (nM)	B _{max} (fmol/mg)	K _d (nM)	B _{max} (fmol/mg)
A ₁ AR	7.33 ± 0.89	2011 ± 85	-	-
β ₁ R	-	-	188 ± 9	15,502 ± 1212
β ₂ R	-	-	48 ± 8	3320 ± 635
A ₁ AR/β ₁ R	7.72 ± 0.24	1020 ± 59 [*]	120 ± 15 [*]	7714 ± 533 [*]
A ₁ AR/β ₂ R	9.87 ± 2.28	1250 ± 202 [*]	13 ± 2 [*]	1120 ± 50 [*]

Data represent means ± SEM (*n* = 3–4). –, no specific binding.^{*} P < 0.05, single receptor transfection vs. co-expression.