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Heterodimerization of α_{2A}- and β_1-Adrenergic Receptors*

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β- and α_2–adrenergic receptors are known to exhibit substantial cross-talk and mutual regulation in tissues where they are expressed together. We have found that the β_1-adrenergic receptor (β_1AR) and α_{2A}-adrenergic receptor (α_{2A}AR) heterodimerize when coexpressed in cells. Immunoprecipitation studies with differentially tagged β_1AR and α_{2A}AR expressed in HEK-293 cells revealed robust co-immunoprecipitation of the two receptors. Moreover, agonist stimulation of α_{2A}AR was found to induce substantial internalization of coexpressed β_1AR, providing further evidence for a physical association between the two receptors in a cellular environment. Ligand binding assays examining displacement of [^{3}H]dihydroalprenolol binding to the β_1AR by various ligands revealed that β_1AR pharmacological properties were significantly altered when the receptor was coexpressed with α_{2A}AR. Finally, β_1AR/α_{2A}AR heterodimerization was found to be markedly enhanced by a β_1AR point mutation (N15A) that blocks *N*-linked glycosylation of the β_1AR as well as by point mutations (N10A/N14A) that block *N*-linked glycosylation of the α_{2A}AR. These data reveal an interaction between β_1AR and α_{2A}AR that is regulated by glycosylation and that may play a key role in cross-talk and mutual regulation between these receptors.

The physiological actions of epinephrine and norepinephrine are mediated via the activation of the following three distinct classes of G protein-coupled receptors (GPCR)[1]: α_1-, α_2-, and β-adrenergic receptors. Each class of adrenergic receptor (AR) is comprised of three closely related subtypes as follows: α_{1A}-, α_{1B}-, and α_{1D}AR, which couple primarily to G_q to stimulate phospholipase activity; α_{2A}-, α_{2B}-, and α_{2C}AR, which couple primarily to G_i to inhibit adenylyl cyclase activity; and β_1-, β_2-, and β_3AR, which couple primarily to G_s to stimulate adenylyl cyclase activity (1). The adrenergic receptor subtypes are differentially distributed across various tissues, and tissue responses to epinephrine and norepinephrine are believed to be dependent upon the relative ratios of the various adrenergic receptors they express.

Because β- and α_2-adrenergic receptors couple to G proteins with opposing actions on adenylyl cyclase activity, the two receptors might be expected to purely antagonize each other's signaling when they are co-stimulated in the same cell. However, it has been shown that α_2AR co-stimulation can in some cases paradoxically sensitize β-adrenergic signaling in brain tissue (2–4). Moreover, the pharmacological properties of βARs in brain tissue are known to be regulated by α_2ARs (5, 6), and reciprocally the pharmacological properties of α_2ARs in brain tissue are known to regulated by βARs (7, 8). These examples of cross-talk and mutual regulation between β- and α_2-adrenergic receptors have been well known for more than 20 years, but the underlying molecular mechanisms remain unclear.

GPCRs have traditionally been thought to exist as monomers, but recent studies (9) have revealed that they can exist in the plasma membrane as both homodimers and heterodimers. At present, a key question in this field is: how widespread is the phenomenon of receptor heterodimerization? The most clear-cut case of the importance of GPCR heterodimerization comes from the $GABA_B$ receptor, a pharmacologically defined entity that is now known to be comprised of two distinct GPCRs, $GABA_BR1$ and $GABA_BR2$ (10). Because $GABA_BR1$ and $GABA_BR2$ are not functional when expressed by themselves, they represent a clear example of the physiological importance of receptor heterodimerization. Although other heptahelical receptors may not absolutely require heterodimerization to be functional in the same way that the $GABA_B$ receptor does, heterodimerization of other receptors may underlie some phenomena that are major question marks in our present understanding of neurotransmitter and hormone receptors, such as unexplained forms of cross-talk between different receptor subtypes.

We wondered if the previously reported cross-talk between βARs and α_2ARs in brain tissue might be due in part to a physical association between these two receptor types. Many early studies (11–13) of GPCR dimerization focused on the β_2AR. We have found recently (14) that the β_1AR also exhibits robust homodimerization in cells. Furthermore, it has been shown recently (15) that β_1AR and β_2AR can heterodimerize in a functionally important manner. β_1AR is the most abundantly expressed βAR in brain (16, 17), a tissue where α_2ARs are found at particularly high levels (18). The most widely expressed α_2AR subtype, α_{2A}AR, is known to be localized both pre- and post-synaptically in a number of brain regions (18), where its pattern of expression overlaps significantly with that of the β_1AR (17). Based on the previously reported functional interactions between α_2ARs and βARs, as well as the overlapping distribution patterns of α_{2A}AR and β_1AR, we examined the possibility that β_1AR might be able to heterodimerize with

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; AR, adrenergic receptor; GABA, γ-aminobutyric acid; PSD-95, post-synaptic density protein of 95 kDa; MAGI, membrane-associated guanylate kinase-like protein with an inverted domain structure; HA, hemagglutinin; HEK, human embryonic kidney; DHA, dihydroalprenolol; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

α_{2A}AR. Our findings reveal that β_1AR and α_{2A}AR robustly associate in cells and that α_{2A}AR can regulate β_1AR internalization and ligand binding.

MATERIALS AND METHODS

Plasmids—FLAG-β_1AR was kindly provided by Robert J. Lefkowitz (Duke University). HA-α_{2A}AR was kindly provided by Lee Limbird (Vanderbilt University Medical Center). HA-β_1AR was kindly provided by Hitoshi Kurose (University of Tokyo). The N15A mutant β_1AR was prepared via PCR amplification from the native human β_1AR cDNA using a mutant sequence oligonucleotide (CTG GGC GCC TCC GAG CCC GGT GCC CTG TCG TCG GCC GCA CCG CTC). The N10A/N14A mutant α_{2A}AR was also prepared via PCR amplification from the wild-type construct in a two-step process, first using a mutant sequence oligonucleotide (CC CTG CAG CCG GAA GCG GGC GCC GCG AGC TGG AAT GGG ACA GAG G) to make the N10A mutation, and second using a second oligonucleotide (GCG GGC GCC GCG AGC TGG GCT GGG ACA GAG GCG CCG GGG GGC) to make the N14A mutation using the N10A mutant construct as a template. The point mutations were confirmed by ABI sequencing.

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from Invitrogen. HEK-293 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO_2 incubator. For heterologous expression of receptors, 2 μg of DNA was mixed with LipofectAMINE (15 μl) and Plus reagent (20 μl) (from Invitrogen) and added to 5 ml of serum-free medium in 10-cm tissue cultures plates containing cells at ~50–80% confluency. Following a 4-h incubation, the medium was removed, and 10 ml of fresh complete medium was added. After another 12–16 h, the medium was changed again, and the cells were harvested 24 h later.

Western Blotting—Samples (5 μg per lane) were run on 4–20% SDS-PAGE gels (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in "blot buffer" (2% non-fat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The primary antibodies utilized were either a 12CA5 monoclonal anti-HA antibody (Roche Molecular Biochemicals) or an M2 monoclonal anti-FLAG antibody (Sigma). The blots were then washed three times with 10 ml of blot buffer and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Biosciences.

Immunoprecipitation—Cells were harvested and lysed in 500 μl of ice-cold lysis buffer (10 mM Hepes, 50 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and the protease inhibitor mixture from Roche Molecular Biochemicals). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 30 μl of beads covalently linked to anti-FLAG antibodies (Sigma) for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 ml of lysis buffer, the immunoprecipitated proteins were eluted from the beads with 1× SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to Western blot analyses.

Enzymatic Deglycosylation—For enzymatic deglycosylation of receptors, immunoprecipitates were separated from beads by boiling for 10 min in a denaturing buffer (0.5% SDS containing 1% β-mercaptoethanol). After cooling, Nonidet P-40 was added to the supernatants to a final concentration of 1%, and Na_2HPO_4/NaH_2PO_4 buffer (pH 7.5) was added to lysates to a final concentration of 50 mM. *N*-glycosidase F (1,500 units; New England Biolabs) was added to a 30-μl reaction volume, and the sample was incubated for 1 h at 37 °C.

Cyclic AMP Assay—Intracellular cAMP was measured by using a non-acetylation cAMP enzyme immunoassay kit (Amersham Biosciences). Briefly, cultured cells were transfected with either FLAG-β_1AR alone or FLAG-β_1AR/HA-α_{2A}AR in combination. After 24 h, cells were split into 6-well culture dishes with fresh medium. After another 48 h, cells were treated with varying concentrations of isoproterenol for 10 min and harvested with cell harvest buffer (50 mM Tris, pH 7.4, 250 μM Ro 20-1724 (Tocris, Ellisville, NJ), 5 mM $MgCl_2$, 1 mM ATP, and 1 μM GTP). Cell lysates were sonicated, transferred to a 96-well assay plate coated with anti-rabbit IgG, and incubated with an anti-cAMP antibody at 4 °C for 2 h along with a series of cAMP standards. A cAMP-peroxidase conjugate was then added to the microtiter plate and incubated at 4 °C for 1 h. The plate was then washed four times with 400 μl of wash buffer, and the wells were incubated with 150 μl of enzyme substrate at room temperature for 1 h. When the samples were within the linear range of the standards, the reaction was stopped by adding 100 μl of 1.0 M sulfuric acid. Absorbance was determined in a plate reader at 450 nm, and cAMP levels were determined using standard curves.

Surface Expression Assay—Transfected cells were split into 35-mm dishes, grown for 48 h, and then incubated in the absence and presence of agonist for 10 min. The cells were then rinsed in PBS and fixed with 4% paraformaldehyde in PBS for 30 min and then rinsed three times in PBS and blocked with blocking buffer (2% non-fat dry milk in PBS, pH 7.4) for 30 min. The fixed cells were then incubated with primary antibody in blocking buffer for 1 h at room temperature. The dishes were subsequently washed three times with 2 ml of block buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) in blocking buffer. Finally, the dishes were washed three times with 2 ml of blocking buffer and one time with 2 ml of PBS and then incubated with 2 ml of ECL reagent (Pierce) for exactly 15 s. The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by placing the plate inside a TD 20/20 luminometer (Turner Designs).

Ligand Binding Assays—For preparation of membranes to be used in ligand binding assays, transfected cells grown on 100-mm dishes were rinsed twice with 10 ml of PBS and then scraped into 1 ml of ice-cold binding buffer (10 mM Hepes, 1 mM $MgCl_2$, 1 mM ascorbic acid, pH 7.4). Cells were then washed three times with 1 ml of binding buffer, sonicated for 10 s, and resuspended in fresh binding buffer for use in radioligand binding assays. Membranes were incubated with increasing concentrations of [^{3}H]DHA or [^{3}H]RX821002 in binding buffer for saturation binding studies, or with 1 nM [^{3}H]DHA or [^{3}H]RX821002 in binding buffer in the absence or presence of various unlabeled ligands to generate inhibition curves. The samples were incubated for 15 min at 37 °C. Nonspecific binding was defined as [^{3}H]DHA or [^{3}H]RX821002 binding in the presence of either 1 mM isoproterenol or 1 mM clonidine, respectively, and represented less than 10% of total binding in all experiments. Incubations were terminated via filtration through GF/C filter paper using a Brandel cell harvester. Filters were rapidly washed three times with ice-cold wash buffer (10 mM Hepes), and radioactive ligand retained by the filters was quantified via liquid scintillation counting. The fitting of curves for one site *versus* two sites was performed using Prism software (GraphPad, San Diego, CA). Goodness of fit was quantified using *F* tests, comparing sum-of-squares values for the one-site *versus* two-site fits.

Immunofluorescence Microscopy—HEK-293 cells were transiently transfected with pcDNA3/FLAG-β_1AR and pcDNA3/HA-α_{2A}AR. Forty eight hours after transfection, cells were washed three times with Dulbecco's PBS and then incubated for 10 min at 37 °C in the absence or presence of 10 μM isoproterenol or 10 μM UK 14,304 (Sigma). Following this incubation, cells were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. To visualize the subcellular localization of β_1AR and α_{2A}AR, cells were blocked and permeabilized with a buffer containing 2% bovine serum albumin and 0.04% saponin in PBS ("saponin buffer") for 30 min at room temperature. The cells were then incubated with anti-β_1AR polyclonal antibody (Santa Cruz Biotechnology) at 1:500 dilution and anti-HA monoclonal antibody (12CA5; Roche Molecular Biochemicals) at 1:1000 dilution for 1 h at room temperature. After three washes (1 min) with saponin buffer, the cells were incubated with a rhodamine red-conjugated anti-rabbit IgG at 1:200 dilution and FITC-conjugated anti-mouse IgG at 1:200 dilution (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After three washes (1 min) with saponin buffer and one wash with PBS, coverslips were mounted, and rhodamine red-labeled β_1AR and FITC-labeled α_{2A}AR were visualized with a Zeiss LSM-410 laser confocal microscope. Multiple control experiments, utilizing either transfected cells in the absence of primary antibody or untransfected cells in the presence of primary antibody, revealed a very low level of background staining, indicating that the primary antibody-dependent immunostaining observed in the transfected cells was specific.

RESULTS

Co-immunoprecipitation of α_{2A}- and β-Adrenergic Receptors—To assess the potential physical association of α_{2A}- and β-adrenergic receptors, HA-α_{2A}AR was expressed in HEK-293 cells either alone or in combination with FLAG-β_1AR or FLAG-

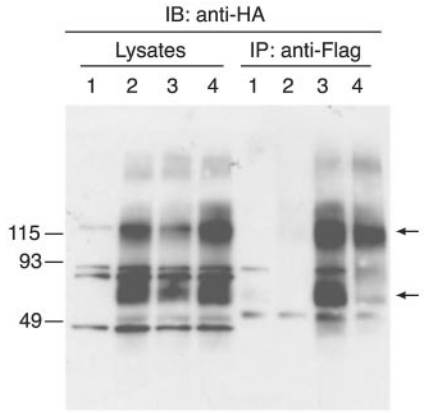

FIG. 1. **Co-immunoprecipitation of α_{2A}- and β_1AR and β_2AR.** HEK-293 cells were transfected with empty vector (*lane 1*), HA-α_{2A}AR alone (*lane 2*), HA-α_{2A}AR/FLAG-β_1AR (*lane 3*), or HA-α_{2A}AR/FLAG-β_2AR (*lane 4*). The expression of HA-α_{2A}AR in detergent-solubilized lysates prepared from the transfected cells is shown in the *1st 4 lanes* of the Western blot (*IB*) shown in this figure. Several nonspecific bands were evident in untransfected cell lysates (*lane 1*), whereas specific immunoreactivity for HA-α_{2A}AR (*lanes 2–4*) was observed as major bands at ~65 and 120 kDa (*arrows*). The lysates were incubated with anti-FLAG affinity agarose to immunoprecipitate the FLAG-tagged β-adrenergic receptors, and the resultant immunoprecipitates (*IP*) were examined via Western blot for anti-HA immunoreactivity. As shown in the *last 2 lanes* of this figure, specific co-immunoprecipitation of HA-α_{2A}AR was observed with both FLAG-β_1AR and FLAG-β_2AR. The positions of molecular mass standards are indicated on the *left side* of the figure. This experiment was repeated five times with nearly identical results.

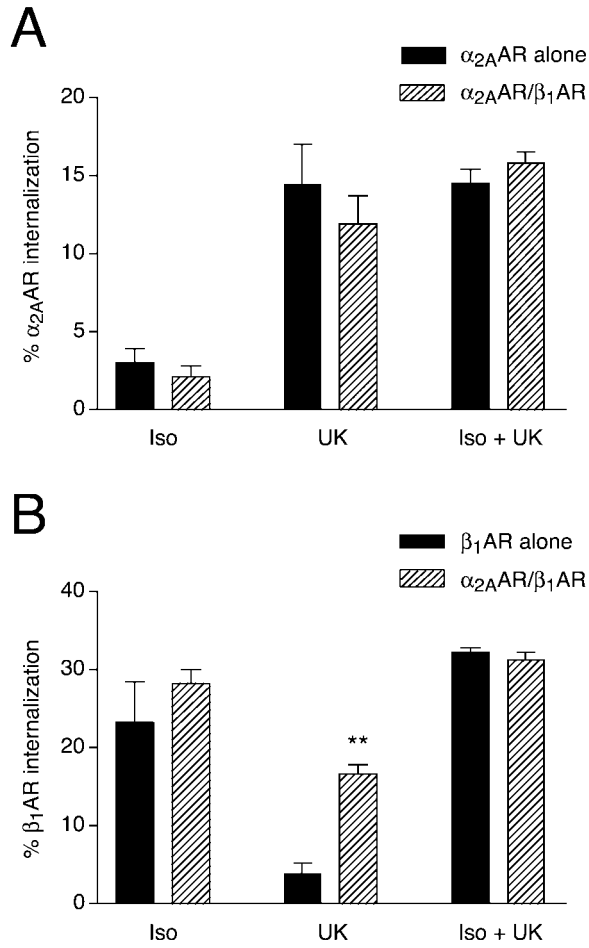

FIG. 2. **Co-internalization of β_1AR with α_{2A}AR.** HA-α_{2A}AR and FLAG-β_1AR were expressed either separately (*solid bars*) or together (*striped bars*) in HEK-293 cells. The internalization of α_{2A}AR (*A*) and β_1AR (*B*) was examined using a luminometer-based assay following 10-min stimulations with the β-adrenergic agonist isoproterenol (*Iso*; 10 μm), the α_2-adrenergic agonist UK 14,304 (*UK*; 10 μM), or a combination of the two agonists together. As shown in *A*, α_{2A}AR exhibited ~15% internalization in response to UK stimulation but no significant internalization in response to isoproterenol under any condition. Conversely, as shown in *B*, β_1AR exhibited ~25–30% internalization in response to isoproterenol but also exhibited ~15% internalization in response to stimulation with UK. This effect was only observed, however, when α_{2A}AR was coexpressed (** indicates significantly different from β_1AR alone, $p < 0.01$). These data suggest that β_1AR can co-internalize with agonist-activated α_{2A}AR. The *bars* and *error bars* represent the means ± S.E. for 4–5 independent experiments for each condition, with each experiment being performed in triplicate.

β_2AR. As shown in Fig. 1, Western blotting for HA-α_{2A}AR in cell lysates revealed multiple bands, with major species at ~65 and 120 kDa. The higher order bands presumably represent receptor complexes resistant to separation by SDS-PAGE, as is commonly observed for many GPCRs (9). When the FLAG-tagged βARs were immunoprecipitated with an anti-FLAG antibody, the co-transfected HA-α_{2A}AR was robustly co-immunoprecipitated. All of the bands of HA-α_{2A}AR immunoreactivity were evident in FLAG-βAR immunoprecipitates. Somewhat more co-immunoprecipitation was observed with β_1AR than with β_2AR, and thus further experiments in this area focused on the β_1AR/α_{2A}AR interaction. No changes in the extent of co-immunoprecipitation were observed when cells were stimulated before harvesting with various adrenergic receptor agonists (data not shown). In related control experiments, HA-α_{2A}AR and FLAG-β_1AR were transfected separately into different plates of cells, which were harvested, prepared as detergent-solubilized lysates, and then mixed together. Immunoprecipitation of FLAG-β_1AR in these experiments did not yield any detectable co-immunoprecipitation of HA-α_{2A}AR (data not shown), revealing that the two receptors need to be expressed in the same cell in order to physically associate.

Co-internalization of α_{2A}- and β_1-Adrenergic Receptors—As a second method of assessing the physical association between α_{2A}AR and β_1AR, we expressed the two receptors in cells and studied their co-internalization. Agonist stimulation of many GPCRs induces significant internalization from the cell surface, and this process is known to be important in the desensitization and resensitization of GPCR responses (19). HA-α_{2A}AR and FLAG-β_1AR were expressed either separately or together in HEK-293 cells and then stimulated with one of three agonist conditions: the βAR agonist isoproterenol alone ("Iso"), the α_2AR agonist UK 14,034 alone ("UK"), or Iso + UK together. When endocytosis of α_{2A}AR was examined via a quantitative luminometer-based assay (Fig. 2*A*), no significant internalization was observed in response to Iso under any condition, whereas internalization in response to UK was ~15% whether Iso was co-applied or not. When endocytosis of β_1AR was examined (Fig. 2*B*), ~25–30% receptor internalization was observed in response to Iso. The extent of internalization was not significantly different for β_1AR expressed alone as compared with β_1AR expressed in the presence of α_{2A}AR. In response to UK, no significant internalization was observed for β_1AR expressed alone, which is the expected result because UK does not activate β_1AR. Strikingly, however, β_1AR coexpressed with α_{2A}AR exhibited ~15% internalization in response to UK stimulation. These data indicate that stimulation of α_{2A}AR can cause co-internalization of β_1AR.

The internalization of α_{2A}AR and β_1AR was also studied via immunofluorescence confocal microscopy. In cells co-transfected with HA-α_{2A}AR and FLAG-β_1AR, immunostaining for both receptors was concentrated into a smooth rim along the edge of the cells, which presumably corresponds to receptor

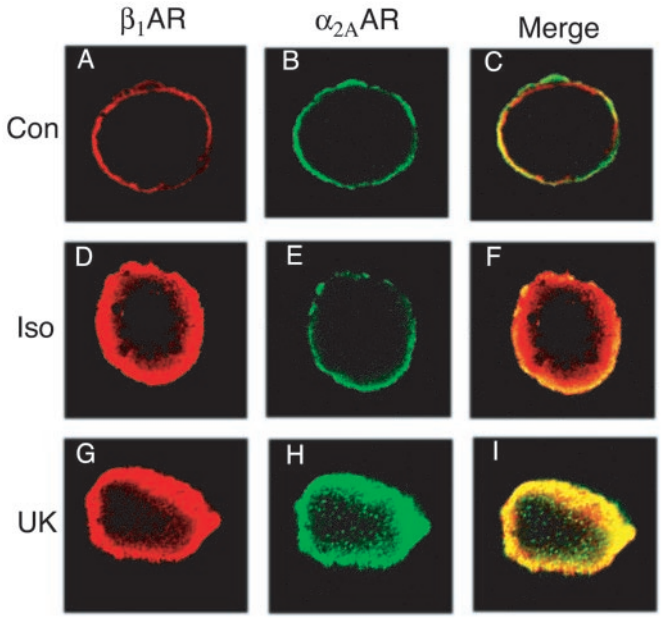

FIG. 3. **Immunofluorescence confocal microscopy reveals agonist-promoted co-internalization of α_{2A}- and β_1-adrenergic receptors.** HA-α_{2A}AR (*red*) and FLAG-β_1AR (*green*) were co-transfected into HEK-293 cells and visualized using secondary antibodies coupled to rhodamine and FITC, respectively. In the absence of agonist stimulation, immunostaining for both receptors was found predominantly in the plasma membrane (*A–C*). Stimulation with isoproterenol (*Iso*) for 10 min induced significant mobilization of β_1AR inside the cell (*D*) but had no significant effect on the subcellular distribution of α_{2A}AR (*E* and *F*). Stimulation with UK 14,034 (*UK*), in contrast, resulted in significant internalization of both α_{2A}AR (*H*) and β_1AR (*G*) and marked co-localization of the two receptors in intracellular regions (*I*, with co-localization indicated in *yellow*). The specificity of staining was determined in control (*Con*) experiments using both untransfected and transfected cells incubated in the absence and presence of the relevant primary antibodies. These data are representative of 3–5 experiments for each condition.

localization in the plasma membrane of the cells (Fig. 3, *A–C*). Stimulation with Iso resulted in the development of significant intracellular immunostaining for FLAG-β_1AR (Fig. 3*D*) but had no apparent effect on the pattern of immunostaining for HA-α_{2A}AR (Fig. 3*E*). In contrast, stimulation with UK resulted in mobilization of both HA-α_{2A}AR and FLAG-β_1AR inside the cell (Fig. 3, *G* and *H*), where the two receptors exhibited significant co-localization (Fig. 3*I*). These data are consistent with the findings obtained using the luminometer-based assay (Fig. 2) and offer additional evidence that α_{2A}AR and β_1AR co-internalize from the cell surface following stimulation with α_2-adrenergic agonists.

Ligand Binding and Signaling of Coexpressed α_{2A}- and β_1-Adrenergic Receptors—Because coexpressed α_{2A}AR was able to regulate β_1AR internalization, we next examined if coexpression with α_{2A}AR was able to regulate β_1AR pharmacological properties. The binding of the βAR-selective antagonist [^{3}H]DHA to lysed membranes derived from cells transfected with either β_1AR alone or β_1AR/α_{2A}AR was examined. Saturation binding studies revealed that [^{3}H]DHA bound with comparable affinity to β_1AR expressed in the absence and presence of α_{2A}AR coexpression (K_D = 2.8 ± 0.5 nM, B_{max} = 39.3 ± 7.7 pmol/mg for β_1AR alone; K_D = 2.3 ± 0.4 nM, B_{max} = 33.0 ± 6.5 pmol/mg for β_1AR/α_{2A}AR). However, studies examining the displacement of [^{3}H]DHA binding by a variety of βAR-selective ligands revealed that many of these compounds exhibited altered affinity for β_1AR coexpressed with α_{2A}AR relative to β_1AR expressed alone. Inhibition curves for displacement of [^{3}H]DHA binding to membranes expressing β_1AR alone were

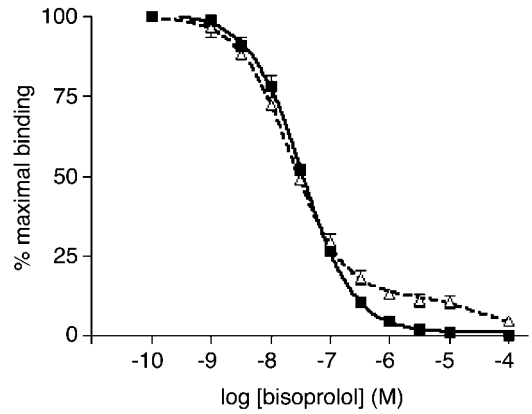

FIG. 4. **Coexpression with α_{2A}AR alters β_1AR pharmacological properties.** HEK-293 cells were transfected with either FLAG-β_1AR alone (*filled squares, solid line*) or HA-α_{2A}AR/FLAG-β_1AR (*open triangles, dashed line*). Membranes were prepared, and the binding of the β-adrenergic antagonist [^{3}H]DHA was studied in the presence of increasing concentrations of the β-adrenergic antagonist bisoprolol. The apparent binding affinity of bisoprolol decreased in the presence of α_{2A}AR coexpression (please see Table I for a summary of other ligands examined in these experiments). Notably, the curve for bisoprolol inhibition of [^{3}H]DHA binding was well fit by assuming a single binding site in the case of β_1AR alone but was a significantly better fit by a two-site analysis in the case of the β_1AR/α_{2A}AR co-transfected samples. The *points* and *error bars* shown are the means ± S.E. for 4 independent determinations each.

fit extremely well by assuming one binding site (Fig. 4; Table I). In contrast, curves for displacement of [^{3}H]DHA binding to membranes expressing β_1AR/α_{2A}AR were in most cases fit significantly better by two-site analyses rather than one-site analyses. The appearance of a significant low affinity component for the displacement of [^{3}H]DHA by metoprolol, labetalol, bisoprolol, dobutamine, and isoproterenol suggests that these ligands bind with substantially lower affinity to β_1AR/α_{2A}AR heterodimers than to β_1AR alone. On the other hand, norepinephrine, an endogenous agonist for both α- and β-adrenergic receptors, exhibited slightly *enhanced* affinity for binding to the β_1AR in the presence of α_{2A}AR coexpression as compared with β_1AR expressed alone, whereas epinephrine, which is also an endogenous agonist for both receptors, exhibited no significant change in its apparent affinity for β_1AR alone *versus* β_1AR/α_{2A}AR. In control experiments, membranes derived from cells expressing β_1AR alone and α_{2A}AR alone were mixed together, as in the control co-immunoprecipitation experiments described above. In these mixing experiments, no changes in the ligand binding properties of β_1AR were observed for any of the ligands examined (data not shown), suggesting that β_1AR and α_{2A}AR must be expressed in the same cell for the modulation of β_1AR pharmacological properties to occur. Furthermore, the effects of α_{2A}AR coexpression on β_1AR ligand binding properties were not blocked by treatment of the cells with pertussis toxin prior to harvesting (data not shown), suggesting that these effects are not due to activation of G_i/G_o-dependent intracellular signaling pathways by the coexpressed α_{2A}AR. In related experiments, the binding of various α_2AR-selective ligands to membranes expressing α_{2A}AR alone *versus* β_1AR/α_{2A}AR was examined. No differences in the binding properties of the α_2AR-selective agonist UK 14,034, the α_2AR-selective partial agonist clonidine, or the α_2AR-selective partial agonist guanfacine were observed (Table II), indicating that although β_1AR possesses altered pharmacological properties when expressed in the presence of α_{2A}AR, it does not seem to reciprocally be the case that α_{2A}AR possesses altered pharmacological properties when expressed in the presence of β_1AR.

We next examined the ability of α_{2A}AR to modulate β_1AR

TABLE I

Ligand binding properties of β_1AR expressed in the absence and presence of α_{2A}AR

The binding of [^{3}H]DHA to lysed membranes was studied in the presence of increasing concentrations of various adrenergic receptor ligands. The estimated K_i values (in nM) are shown for each ligand. One-site and two-site fits of each data set were performed as described under "Materials and Methods." The inhibition curves for β_1AR/α_{2A}AR were significantly better fit by two-site fits rather than one-site fits for all ligands except for propranolol, norepinephrine, and epinephrine, whereas the inhibition curves for β_1AR alone were not significantly better fit in any case by two-site fits relative to one-site fits. Hence, two K_i values (K_H for the high affinity component and K_L for the low affinity component) are provided for binding to β_1AR/α_{2A}AR for most of the ligands, whereas only a single K_i value is provided for binding to β_1AR expressed alone. Note that for all of the two-site fits, the K_H value for binding to β_1AR/α_{2A}AR is similar to the single K_i value for binding to α_{2A}AR alone, suggesting that the majority of binding sites in the membranes expressing β_1AR/α_{2A}AR possess binding properties similar to the binding sites in membranes expressing β_1AR alone. However, membranes expressing β_1AR/α_{2A}AR also exhibit, in most cases, a small low affinity component (K_L), which was estimated between 10 and 25% of total binding sites in all cases, as shown in the right-hand column. The data for these inhibition curves were derived from 3 to 5 independent determinations for each ligand.

	β_1AR, K_i	β_1AR/α_{2A}AR		
		K_H	K_L	K_L
	nM	nM	nM	%
βAR-selective ligands				
Bisoprolol	24 ± 6	16 ± 5	22,190 ± 7748	10
Dobutamine	1609 ± 97	1849 ± 417	40,360 ± 9620	22
Isoproterenol	514 ± 46	267 ± 81	12,170 ± 2321	15
Labetalol	36 ± 8	25 ± 10	11,250 ± 1773	12
Metoprolol	93 ± 5	88 ± 19	13,380 ± 4345	11
Propranolol	5.7 ± 1.6	7.6 ± 1.4		
Endogenous ligands				
Epinephrine	5562 ± 578	5564 ± 492		
Norepinephrine	5010 ± 701	3799 ± 404		

TABLE II

Ligand binding properties of α_{2A}AR expressed in the absence and presence of β_1AR

The binding of [^{3}H]RX821002 to lysed membranes was studied in saturation binding studies, and no significant differences were found for α_{2A}AR/β_1AR relative to α_{2A}AR alone (K_D = 6.0 ± 2.1 nM, B_{max} = 7.7 ± 1.8 pmol/mg for α_{2A}AR alone; K_D = 4.6 ± 1.5 nM, B_{max} = 6.8 ± 1.6 pmol/mg for α_{2A}AR/β_1AR). The binding of [^{3}H]RX821002 to lysed membranes was also studied in the presence of increasing concentrations of several other α-adrenergic receptor ligands. The estimated K_i values (in nM) are shown for each ligand. The levels of significance of differences in ligand binding to α_{2A}AR/β_1AR relative to α_{2A}AR alone were assessed via *t* tests, and no significant differences were found between any of the matched sets. Moreover, one-site *versus* two-site fits were performed as described under "Materials and Methods" and in no cases were two-site fits significantly better than one-site fits. Thus, α_{2A}AR ligand binding properties showed no obvious differences when α_{2A}AR was examined in the absence and presence of β_1AR coexpression. The data for these inhibition curves were derived from three independent determinations for each ligand.

	α_{2A}AR, K_i	α_{2A}AR/β_1AR, K_i
	nM	
α_2AR-selective ligands		
Clonidine	34 ± 9	33 ± 8
Guanfacine	17 ± 5	26 ± 6
UK 14,304	91 ± 14	89 ± 9

signaling. We utilized a transfection-based approach to study β_1AR stimulation of cAMP production in the absence and presence of α_{2A}AR coexpression in HEK-293 cells. These studies revealed that isoproterenol was significantly less potent at stimulating cAMP production when β_1AR was expressed in the presence of α_{2A}AR than when β_1AR was expressed alone (Fig. 5). The maximal extent of cAMP production, however, was comparable in both cases, and the expression level of β_1AR was unaltered by coexpression of α_{2A}AR. Moreover, the effect of α_{2A}AR coexpression on isoproterenol-induced cAMP production was not attributable to constitutive coupling of α_{2A}AR to G_i, because the effect was not blocked by pertussis toxin treatment (data not shown). These data reveal that isoproterenol has a higher potency at β_1AR expressed alone *versus* β_1AR coexpressed with α_{2A}AR. These findings are consistent with the ligand binding data presented in Table I, which indicate that coexpression with α_{2A}AR results in reduced β_1AR affinity for isoproterenol and other βAR-selective ligands.

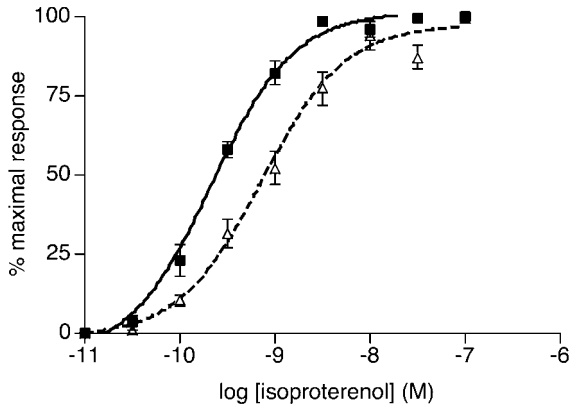

FIG. 5. **Coexpression of β_1AR with α_{2A}AR alters the potency of isoproterenol-induced stimulation of adenylyl cyclase.** HEK-293 cells were transfected with either β_1AR alone (*filled squares, solid line*) or β_1AR/α_{2A}AR (*open triangles, dotted line*). Expression levels of the β_1AR were identical for the two transfection conditions, as assessed by Western blot. The cells were stimulated with increasing concentrations of isoproterenol, and agonist-induced rises in cellular cyclic AMP were quantified. The maximal extent of cyclic AMP produced in the β_1AR/α_{2A}AR cells was 106 ± 8% of that produced in the cells transfected with only β_1AR. The EC_{50} for isoproterenol stimulation of β_1AR alone was 0.16 ± 0.02 nM, as compared with 0.68 ± 0.17 for β_1AR/α_{2A}AR (significantly different from β_1AR alone, $p < 0.01$). The *points* and *error bars* represent the means and S.E. values for four independent determinations.

Regulation of α_{2A}-AR/β_1AR Heterodimerization by Receptor Glycosylation—Glycosylation of G protein-coupled receptors can have variable effects on receptor trafficking and signaling (20). The β_1AR contains one consensus site for *N*-linked glycosylation on its extracellular amino terminus (Asn-15). We mutated this site to alanine, creating a mutant receptor (N15A) that exhibited a significant decrease in apparent size on SDS-PAGE (Fig. 6*A*). Enzymatic deglycosylation with *N*-glycosidase F also decreased the apparent size of the wild-type β_1AR on SDS-PAGE but had no effect on the apparent size of the N15A mutant receptor suggesting that Asn-15 is the sole site of β_1AR *N*-linked glycosylation (21). We examined the capacity of the N15A mutant receptor for heterodimerization with the α_{2A}AR. HA-α_{2A}AR was coexpressed with either FLAG-β_1AR wild-type or FLAG-β_1AR N15A, which exhibited equivalent levels of total

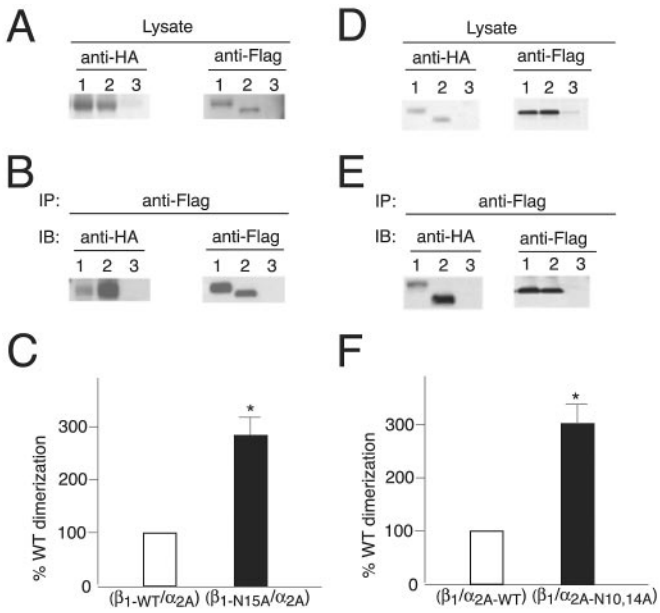

FIG. 6. **Heterodimerization of α_{2A}AR and β_1AR is enhanced when receptor glycosylation is blocked.** HEK-293 cells were transfected with HA-α_{2A}AR and FLAG-β_1AR wild-type (*lane 1*), HA-α_{2A}AR wild-type and the FLAG-β_1AR N15A mutant (*lane 2*), or empty vectors (*lane 3*). The total expression levels of all the transfected proteins are shown in *A*. For the anti-FLAG-β_1AR samples (*right*), note the decrease in the apparent size of the band for the N15A mutant, corresponding to the decreased glycosylation of this mutant receptor. For both wild-type and N15A mutant β_1AR, several higher order immunoreactive bands were evident in transfected cell lysates, but only the lowest molecular weight band (~54 kDa) is shown here to demonstrate the comparable levels of expression of the wild-type and mutant receptors. The transfected cells were harvested, solubilized, and incubated with anti-FLAG affinity resin to immunoprecipitate FLAG-β_1AR. The resultant immunoprecipitates (*IP*) were run on 4–20% SDS-PAGE gels and probed on Western blots (*IB*) to detect both anti-HA (*B, left blot*) and anti-FLAG (*B*, *right blot*) immunoreactivity. The FLAG-tagged wild-type and N15A mutant β_1-adrenergic receptors were immunoprecipitated equally (*B, right blot*). The amount of HA-α_{2A}AR that was co-immunoprecipitated (*B*, *left blot*) differed markedly, however, with the N15A mutant pulling down an average of nearly 3-fold more HA-α_{2A}AR than the wild-type β_1AR. These data reveal that β_1AR/α_{2A}AR heterodimerization is enhanced for the N15A mutant β_1AR relative to wild-type β_1AR. Quantification of all of these data is shown in *C*. Similar experiments were performed in the experiments illustrated in *D–F*, except that the three lanes that are shown correspond to HA-α_{2A}AR and FLAG-β_1AR wild-type (*lane 1*), HA-α_{2A}AR N10A/N14A mutant and FLAG-β_1AR wild-type (*lane 2*), and empty vectors (*lane 3*). Blockade of α_{2A}AR glycosylation, like blockade of β_1AR glycosylation, resulted in enhanced heterodimerization between the two receptors. The *bars* and *error bars* shown in *C* and *F* represent the means ± S.E. for 4 independent determinations for each condition. *WT*, wild type.

cellular expression as shown in Fig. 6*A*. The FLAG-tagged β_1ARs were immunoprecipitated with an anti-FLAG antibody, and the amount of co-immunoprecipitated HA-α_{2A}AR was examined via Western blot (Fig. 6*B*) and quantified (as shown in Fig. 6*C*). Strikingly, α_{2A}AR was co-immunoprecipitated much more efficiently with the N15A mutant β_1AR than with the wild-type β_1AR. These data reveal that blockade of β_1AR glycosylation enhances β_1AR heterodimerization with α_{2A}AR.

Because α_{2A}AR/β_1AR heterodimerization was more efficient for the N15A mutant, we examined whether or not α_{2A}AR might exert a more robust regulation of N15A-β_1AR relative to wild-type β_1AR. However, we found that agonist-promoted β_1AR internalization following a 10-min stimulation with UK 14,304 was not significantly different for wild-type β_1AR *versus* the N15A mutant (wild type = 16.8 ± 3.5%; N15A = 18.0 ± 4.4% in matched plates examined side-by-side; $n = 3$). Similarly, the changes in ligand binding properties induced by coexpression with α_{2A}AR were comparable for the wild-type β_1AR and the N15A mutant (data not shown). Thus, although blockade of β_1AR glycosylation results in a clear enhancement of α_{2A}AR/β_1AR heterodimerization, it may not lead to an enhancement in heterodimerization of functional receptors on the cell surface. This observation may be related to the fact that the N15A mutant β_1AR is deficient in its ability to traffic to the cell surface relative to the wild-type receptor (21).

The α_{2A}AR is also known to be glycosylated on its amino terminus, on residues Asn-10 and Asn-14 (22). We therefore prepared a mutant version of the α_{2A}AR (N10A/N14A) that cannot be glycosylated. Transfection of this mutant construct into HEK-293 cells resulted in the expression of receptors with significantly decreased apparent size on SDS-PAGE gels relative to wild-type α_{2A}AR (Fig. 6*D*), as reported previously (22). The heterodimerization of the HA-tagged N10A/N14A mutant with wild-type FLAG-β_1AR was assessed in side-by-side experiments in comparison to the heterodimerization of wild-type α_{2A}AR with wild-type FLAG-β_1AR (Fig. 6*E*). The N10A/N14A mutant α_{2A}AR exhibited an ~3-fold enhancement over wild-type α_{2A}AR in heterodimerization with β_1AR (Fig. 6*F*). Taken together with the experiments described above examining the N15A β_1AR, these findings indicate that blockade of glycosylation of both α_{2A}AR and β_1AR results in enhanced α_{2A}AR/β_1AR heterodimerization.

DISCUSSION

Our findings reveal a functionally important heterodimerization between α_{2A}- and β_1-adrenergic receptors. The evidence for the physical association of these two receptor subtypes is derived from both co-immunoprecipitation and co-internalization assays. The co-internalization experiments not only represent evidence for the physical association of the two receptors, they also represent a specific mechanism by which α_{2A}AR stimulation may influence β_1AR function. Although α_2ARs and βARs couple primarily to G proteins with opposing cellular effects on cAMP production, it is known that agonist activation of α_2ARs can in some cases paradoxically sensitize βAR signaling in brain tissue (2–4). Because internalization of GPCRs is known to play a key role in promoting GPCR resensitization (19), our observation that α_{2A}AR stimulation can promote β_1AR internalization provides a specific molecular mechanism that could potentially account for the previously reported ability of α_2AR stimulation to sensitize βAR-mediated responses in native tissues.

Coexpression of α_{2A}AR and β_1AR in our studies not only allowed for α_{2A}AR regulation of β_1AR internalization, it also resulted in altered β_1AR pharmacological properties. We found that the curves for displacement of [^{3}H]DHA binding to membranes expressing β_1AR alone were fit well by one-site analyses for all ligands examined. Whereas it is true that analyses of agonist binding to β-adrenergic receptors often require resolution into two sites, which correspond to G protein-coupled (high affinity) *versus* uncoupled (low affinity) states (23), our analyses of agonist binding to β_1AR alone were fit well by assuming a single site. This is probably due to the fact that the transfected β_1AR was expressed in our cells at much higher levels than the endogenous G proteins, meaning that the G protein-coupled (high affinity) component of agonist binding to the receptors in our assays represented only a tiny and unresolvable component of the inhibition curves. In any case, coexpression of β_1AR with α_{2A}AR resulted in the appearance of a significant low affinity component of binding for many of the βAR-selective ligands. Our interpretation of these data is that a proportion of the β_1AR in the cells assembled with α_{2A}AR to form heterodimers that exhibited unaltered affinity for some ligands (such as DHA and propranolol), substantially reduced

affinity for other ligands (such as metoprolol, labetalol, bisoprolol, isoproterenol and dobutamine), and slightly increased affinity for yet other ligands (such as the endogenous agonist norepinephrine). Many of the ligands examined share significant structural similarity, and it is therefore uncertain why the binding properties of the various ligands should be differentially altered by β_1AR coexpression with α_{2A}AR. Moreover, the affinity constant values derived for the low affinity component of binding in these studies must be considered as rough estimates, because it is difficult to derive accurate affinity constant estimates from curves where the size of the low affinity component (10–25%) represents such a small minority of the total population of binding sites.

A question of interest is why coexpression of β_1AR with α_{2A}AR should result in ligand binding curves that are best fit by two sites, rather than simply resulting in a single population of novel binding sites as might be expected if every β_1AR were to heterodimerize with an α_{2A}AR to form receptors with novel pharmacological properties. The most likely explanation for the observed mixed population of binding sites is that, regardless of how efficiently α_{2A}AR heterodimerizes with β_1AR, it is unlikely that *all* β_1AR in a given cell will form heteromeric complexes with coexpressed α_{2A}AR. A large proportion of cellular β_1-adrenergic receptors are likely to exist either as monomers or homodimers, with only a fraction of the total β_1AR population assembling with other receptors such as α_{2A}AR. Thus, studies examining the binding of ligands to co-transfected α_{2A}AR/β_1AR are almost certainly studying mixed populations of receptors, complicating attempts to estimate the true changes in β_1AR pharmacological properties induced by heterodimerization with α_{2A}AR. This is a general problem shared by all studies examining pharmacological changes induced by heterodimerization of GPCRs.

Over the past several years, heterodimerization of a number of different types of GPCRs has been reported. Examples where heterodimerization is required for the formation of functional receptors include the GABA receptors $GABA_BR1$ and $GABA_BR2$ (10) and the taste receptor combinations T1R1/T1R3 and T1R2/T1R3, which have been reported to form receptors for umami and sweet stimuli, respectively (24–26). Examples where heterodimerization allows for cross-regulation between receptors but is not *required* for receptor function include the following: β_1- and β_2-adrenergic receptors (15); δ and κ opioid receptors (27); δ and μ opioid receptors (28, 29); δ opioid and β_2-adrenergic receptors (30, 31); muscarinic acetylcholine M2 and M3 receptors (32); angiotensin AT1 and bradykinin B2 receptors (33); dopamine D1 and adenosine A1 receptors (34); dopamine D2 and somatostatin SSTR5 receptors (35); dopamine D2 and adenosine A2 receptors (36); mGluR1 glutamate and A1 adenosine receptors (37); SSTR1 and SSTR5 somatostatin receptors (38); SSTR2A and SSTR3 somatostatin receptors (39); and μ opioid and SSTR2A somatostatin receptors (40). Many of these receptor/receptor interactions have been found to result in altered pharmacological properties for one or both receptors (27–29, 32, 35, 38, 39), similar to what we have found for the α_{2A}AR/β_1AR interaction. Additionally, several of the previously reported (30, 36, 40) receptor-receptor interactions have been found to facilitate receptor co-internalization, similar to our observation that stimulation of α_{2A}AR can lead to co-internalization of β_1AR.

In both the luminometer-based surface expression assays and the immunofluorescence microscopy experiments performed on α_{2A}AR/β_1AR co-transfected cells, we observed that α_{2A}AR stimulation resulted in internalization of both α_{2A}AR and β_1AR, whereas stimulation of β_1AR resulted in internalization of only β_1AR. The reason for this difference is not clear. It may be case that the α_{2A}AR/β_1AR heterodimer has internalization properties that are distinct from either of the two individual receptors. Alternatively, α_{2A}AR/β_1AR heterodimerization may be impaired by β-adrenergic agonist stimulation, allowing β_1AR to temporarily internalize in the absence of α_{2A}AR co-internalization. Our co-immunoprecipitation studies, however, did not reveal any consistent effects of agonist stimulation on the amount of HA-α_{2A}AR co-immunoprecipitated with FLAG-β_1AR. It is uncertain, however, whether or not this technique is sensitive enough to detect changes in α_{2A}AR/β_1AR co-immunoprecipitation in the range of 10–20%, as might be expected if receptor internalization were correlated with a temporary release from heterodimerization. One thing that is interesting to note is the similarity between our findings for the effects of α_{2A}AR/β_1AR heterodimerization on receptor ligand binding properties *versus* internalization; we found that assembly with α_{2A}AR influenced β_1AR pharmacological properties, whereas conversely assembly with α_{2A}AR did not result in any evident change in the ligand binding properties of α_{2A}AR. Similarly, we found that α_{2A}AR stimulation led to β_1AR internalization, but conversely β_1AR stimulation did not lead to any evident internalization of α_{2A}AR. Thus, for both ligand binding and internalization, α_{2A}AR was able to influence β_1AR, but β_1AR was not able to influence α_{2A}AR.

If β_1-adrenergic receptors can form β_1AR/β_1AR homodimers (14), β_1AR/β_2AR heterodimers (15), and β_1AR/α_{2A}AR heterodimers, as the present data reveal, it is a point of significant interest to understand the factors that regulate the proportion of cellular homodimers *versus* heterodimers. Studies on the dimerization of other GPCRs have provided evidence that agonist stimulation can regulate dimerization (9). However, as mentioned above, our co-immunoprecipitation experiments did not reveal any consistent effects of agonist stimulation on α_{2A}AR/β_1AR heterodimerization. Association with cytoplasmic scaffold proteins is another factor that might potentially regulate heterodimer formation. The β_1-adrenergic receptor is known to associate with PSD-95/Discs-large/ZO-1 homology domain-containing scaffold proteins such as PSD-95 (14, 41, 42) and MAGI-2 (14). However, we have not observed any significant effects of PSD-95 or MAGI-2 coexpression on the extent of either β_1AR/β_1AR homodimerization (14) or α_{2A}AR/β_1AR heterodimerization (data not shown). The α_{2A}-adrenergic receptor is known to associate with cytoplasmic proteins such as 14-3-3 (43) and spinophilin (44), but we have not examined the effects of these interactions on α_{2A}AR/β_1AR heterodimerization.

One additional way that receptor heterodimerization might be regulated is via post-translational receptor modifications. A post-translational modification common to many GPCRs is receptor glycosylation (20). We have found that the β_1AR is *N*-glycosylated on a single residue, Asn-15, and that α_{2A}AR/β_1AR heterodimerization is markedly *enhanced* via mutation of Asn-15 to an amino acid that cannot be glycosylated. Moreover, we have also found that α_{2A}AR/β_1AR heterodimerization is enhanced by blocking glycosylation of the α_{2A}AR. These data could indicate that lack of glycosylation alters the conformations of the α_{2A}AR and β_1AR such that the efficiency of their heterodimerization is increased. Alternatively, it is possible that the enhanced heterodimerization of the N10A/N14A α_{2A}AR and N15A β_1AR is the result of a more global alteration in the trafficking and processing of the mutant receptors. Interestingly, we have found previously (21) that β_1AR homodimerization is impaired for the β_1AR-N15A mutant relative to the wild-type receptor. Thus, our data indicate that blockade of glycosylation has differential effects on β_1AR homo-*versus* heterodimerization. In any case, it is known that the

glycosylation state of transmembrane receptors can vary significantly in different tissue types (45, 46). The glycosylation state of the β_1AR in particular is known to be regulated via polymorphic variation (47). Thus, the extent of α_{2A}AR/β_1AR heterodimerization may be regulated differentially between tissues and between individuals via differences in β_1AR glycosylation state.

In summary, we have found a physical association between α_{2A}- and β_1-adrenergic receptors. This heterodimerization alters β_1AR pharmacological properties and facilitates cross-internalization of β_1AR following α_{2A}AR agonist stimulation. Both α_{2A}AR and β_1AR are abundantly expressed in the brain, and heterodimerization of these two receptors might therefore underlie previously reported functional cross-talk between endogenous α_2- and β-adrenergic receptors in brain tissue (2–8). Therapeutic drugs acting on α_2ARs (such as clonidine) and β-adrenergic receptors (such as propranolol and metoprolol) are commonly utilized in the treatment of hypertension and are known to exhibit significant clinical interactions (48, 49). The heterodimerization of α_{2A}AR and β_1AR described here may help to provide new insights into both physiological cross-talk between α_2- and β-adrenergic receptors and clinical interactions between therapeutic drugs acting on these receptor subtypes.

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