α_{2A}- and α_{2C}-Adrenergic Receptors Form Homo- and Heterodimers: The Heterodimeric State Impairs Agonist-Promoted GRK Phosphorylation and β-Arrestin Recruitment†

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ABSTRACT: Dimerization of seven transmembrane-spanning receptors diversifies their pharmacologic and physiologic properties. The α_2-adrenergic receptor (α_2AR) subtypes A and C are both expressed on presynaptic nerves and act to inhibit norepinephrine release via negative feedback. However, in vivo and in vitro studies examining the roles of the two individual α_{2A}- and α_{2C}AR subtypes are not readily reconciled. We tested the hypothesis that the receptors form homo- and heterodimers and that the α_{2A}−α_{2C} heterodimer has unique properties. SDS−PAGE of epitope-tagged receptors revealed potential oligomers including dimers. BRET of live HEK-293 cells transfected with the subtypes fused to Rluc or YFP revealed that both subtypes form dimers and the heterodimer. A lower $BRET_{50}$ for the α_{2A}−α_{2C} heterodimer (0.79 ± 0.20) compared to that of the α_{2A} or α_{2C} homodimer (2.331 ± 0.44 or 3.67 ± 0.69, respectively) suggests that when both subtypes are expressed, there is a greater likelihood that the two receptors will form the heterodimer than homodimers. Co-immunoprecipitation studies confirmed homo- and heterodimer formation. The presence of the α_{2C}AR within the heterodimer resulted in a marked reduction in the level of GRK2-mediated α_{2A}AR phosphorylation, which was accompanied by a qualitative attenuation of β-arrestin recruitment. Signaling of the α_{2A}−α_{2C} heterodimer to the β-arrestin-dependent activation of Akt was decreased compared to that of the α_{2A}AR homodimer, while p44/p42 MAP kinase activation was unaffected. Thus, the α_{2C}AR alters α_{2A}AR signaling by forming oligomers, and these complexes, which appear to be preferred over the homodimers, should be considered a functional signaling unit in cells in which both subtypes are expressed.

Three human α_2-adrenergic receptor subtypes (α_2ARs)[1] have been cloned and are denoted α_{2A}, α_{2B}, and α_{2C}. All three subtypes couple to $G_{\alpha i}$ and are activated by the endogenous agonists epinephrine and norepinephrine. Early results from studies of the cloned human receptors did not readily indicate the physiologic basis for the necessity for these distinct subtypes. Subsequent studies, though, have revealed a number of subtype-specific characteristics (*1*−*5*). Of particular interest due to their potential role in neurotransmitter release are the α_{2A}- and α_{2C}AR subtypes, both of which are expressed on cardiac and other presynaptic nerve terminals and regulate release of norepinephrine by negative feedback (*6*). The α_{2A}AR regulates norepinephrine release evoked by high-frequency neuronal stimulation and is thought to represent a mechanism for coordinating intense, acute, sympathetic responses. In contrast, α_{2C}AR modulate norepinephrine release due to low-frequency stimulation and appear to primarily regulate basal, long-term, neurotransmitter release. In cells transfected to individually express α_{2A}- or α_{2C}AR, coupling to $G_{\alpha i}$ (measured as inhibition of adenylyl cyclase) and stimulation of MAP kinase by various agonists is similar between the subtypes (*1*, *7*, *8*). However, several phenotypic differences have been identified which center on agonist-promoted regulation. In transfected cells, the α_{2A}AR undergoes rapid agonist-promoted desensitization of receptor function ($G_{\alpha i}$ coupling) which is due to receptor phosphorylation by GRKs (*2*, *4*). Such phosphorylation occurs at four serines in the third intracellular loop of α_{2A}AR (*9*). GRK2 and GRK3 appear to be responsible for this phosphorylation, as opposed to GRK5 or GRK6 (*10*). In contrast, under the same conditions, α_{2C}AR display no agonist-promoted desensitization or phosphorylation by GRK2, -3, -5, or -6 (*4*, *10*). Of note, when the α_{2C}AR third intracellular loop is substituted into the α_{2A}AR and expressed in cells, the chimera undergoes agonist-promoted phosphorylation and desensitization (*11*). Similarly, phosphorylation of the third intracellular loop of the α_{2A}AR is lost when in

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[1] Abbreviations: AR, adrenergic receptor(s); HA, hemagglutinin; YFP, yellow fluorescent protein; GFP, green fluorescent protein; PBS, phosphate-buffered saline.

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the context of analogous chimeric α_{2C}AR (*11*). This suggests not only that serines or threonines are necessary for GRK-mediated phosphorylation but also that the conformation of the loop containing the phosphate acceptors, dictated in part by the transmembrane backbone, or interaction with other intracellular loops, is important. Once phosphorylated by GRKs, many seven-transmembrane (7-TM) receptors bind one or more of the arrestins, which is a principal mechanism by which GRK-mediated desensitization occurs (*12*). Arrestin recruitment also represents a signaling event via its scaffolding of other proteins (*13*). On the basis of this paradigm, α_{2A}AR would be expected to have greater arrestin binding and/or recruitment than α_{2C}AR. There are subtype-specific differences in agonist-promoted internalization as well, but the extent of the difference between α_{2A}- and α_{2C}AR is highly dependent on coexpression of certain arrestins (*8*). Of note, these phenotypes are potentially confounded by somewhat different subcellular distributions of the two subtypes. In endogenously expressing (*14*, *15*) or transfected (*1*, *4*, *16*) cells, α_{2A}- and α_{2C}AR-mediated inhibition of adenylyl cyclase (as assessed in whole-cell cAMP assays or cell membrane-fraction adenylyl cyclase activity assays) is readily observed. Such studies indicate that both subtypes are expressed on the cell surface and oriented to bind agonist and couple to G-proteins. However, a significant portion of the total α_{2C}AR complement appears to be intracellular, but the extent of the intracellular component may be cell-type- and temperature-dependent (*17*−*19*).

The differences in α_{2A} and α_{2C}AR phosphorylation and desensitization, as assessed in cells transfected to individually express the two subtypes, are not readily reconciled with gene-ablated mouse and human studies. The propensity for acute desensitization of α_{2A}AR seems inconsistent with the need for rapid presynaptic α_{2A}AR action under high neuronal stimulation frequencies, and in fact, α_{2A}AR function as ascertained by norepinephrine release does not appear to undergo desensitization (*6*). Furthermore, in humans with severe heart failure, where NE levels are elevated and marked α_{2A}AR desensitization would be expected, α_{2A}AR function is readily observed in vivo (*20*). In contrast to the α_{2A}AR, recombinantly expressed α_{2C}AR do not undergo GRK-mediated desensitization (*2*, *4*), yet it would seem physiologically necessary for a receptor that controls NE release over long periods of time to have the capacity for regulation in response to various pre- and postsynaptic events to maintain optimal cardiac function. Interestingly, α_{2C}AR knockout mice have normal hearts until they are exposed to acute pressure overload from aortic banding, which results in cardiomyopathy (*21*). Since both α_{2A}- and α_{2C}ARs are coexpressed in the cardiac presynaptic nerve terminal, we have considered the possibility that they form heterodimers and that this complex has characteristics that differ from those of either individual receptor or have "hybrid" functions. In transfected cells, we found that both receptor subtypes form homodimers, as well as the heterodimer, with the latter appearing to be the preferred oligomer. Heterodimerization of the two subtypes results in a decrease in the level of agonist-promoted β-arrestin recruitment and GRK2-mediated α_{2A}AR phosphorylation. Since we found no agonist-promoted β-arrestin recruitment or GRK phosphorylation of the α_{2C}AR, its heterodimerization quenches these two early signaling events by the α_{2A}AR.

EXPERIMENTAL PROCEDURES

Expression Constructs. The human α_{2A} and α_{2C} cDNAs in pcDNA3 were constructed by previously described PCR techniques (*22*, *23*) for expression in-frame, at the amino terminus, of the influenza hemagglutinin (HA) or FLAG sequences, to provide epitopes for immunoprecipitation and Western blotting (see below). These were subsequently utilized to construct in-frame, carboxy-terminal, fusion proteins with Renilla luciferase (Rluc) or yellow fluorescent protein (YFP), by subcloning into PRluc-N3(h) (Perkin-Elmer) or pGFP-CMV-topaz (M. Bouvier, University of Montreal, Montreal, PQ).

Cell Culture and Transfection. Human embryonic kidney (HEK-293) cells were grown in monolayers in DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. In some studies, cells were seeded onto 100 mm plates at a density of 1×10^7 cells/plate in medium lacking penicillin and streptomycin, and transfections with amino epitope-tagged constructs were performed using Lipofectamine (Gibco/BRL), typically at a ratio of 1 μg of DNA per 3 μL of Lipofectamine. For BRET, 48 h after transfection with the Rluc or YFP constructs, cells were detached with Versene (Gibco/BRL), rinsed with PBS, and resuspended in PBS containing 1% glucose and aliquoted to 96-well microplates at a density of ~80000 cells/well.

BRET Measurements. These studies were carried out with a Flexstation II scanning fluorimeter/luminometer (Molecular Devices) in a 96-well format using methods essentially described previously by Bouvier and colleagues (*24*). Briefly, cells were cotransfected with Rluc- and YFP-tagged constructs, and coelenterazine h was added at a final concentration of 5 μM for 15 min prior to light-emission acquisition. Luminescence spectrum scanning from 420 to 560 nm was performed to ascertain the BRET spectrum for each sample, and readings of peak signals at 475 and 530 nm (performed in triplicate) were averaged and used to calculate BRET ratios. In parallel studies, transfections with the Rluc-tagged construct were utilized to acquire the spectrum of luciferase emission alone. The BRET signal was determined by calculating the ratio of the emission by the receptor−YFP construct at 530 nm over the emission by the receptor−Rluc construct at 475 nm, correcting for the background signal of receptor−Rluc constructs expressed alone, using the formula $[(Em_{530} - Em_{475})CF]/Em_{475}$, where $CF = Em_{530}/Em_{475}$ was obtained from cells expressing the receptor-Rluc construct alone in each experiment. In all studies, cells transfected with the receptor−YFP construct were studied with excitation at 475 nm to verify the expected spectra and to calculate the YFP/Rluc values. Maximal BRET levels ($BRET_{max}$) were obtained by cotransfecting increasing amounts of receptor−YFP constructs with a constant quantity of receptor−Rluc construct so that the YFP/Rluc ratio (obtained from the spectral output) incrementally increased. Given that saturation of BRET with increasing YFP/Rluc signals was observed for some α_2AR transfections, the $BRET_{max}$ and $BRET_{50}$ were derived from fitting these data to a one-site hyperbolic function.

SDS−PAGE and Receptor Co-Immunoprecipitation. Cells from a 100 mm dish were washed three times with cold PBS; one-third of the cells were detached and resuspended in 5

mM Tris and 2 mM EDTA (pH 7.40), and membranes were prepared for radioligand binding as described below. The remaining cells were solubilized in 500 μL of lysis buffer [0.5% Triton X-100, 50 mM NaCl, 10 mM HEPES, and 5 mM EDTA (pH 7.40)] containing the protease inhibitors benzamadine, soybean trypsin inhibitor, aprotinin, and leupeptin, by rotation for 4 h at 4 °C. After centrifugation at 20000*g* for 20 min to remove unsolubilized material, the supernatant was utilized for Western blots and co-immunoprecipitation. For studies aimed at initial identification of higher-molecular weight (oligomeric) species, 100 μg of solubilized protein was subjected to 8% SDS−PAGE in the absence of β-mercaptoethanol. Proteins were transferred to PVDF membranes, and Western blots were carried out with a 1:500 dilution of anti-HA antibody clone 3F10 (Roche). Detection was by the ECL Advanced Western Blotting kit (Amersham) which is ~100-fold more sensitive than the standard ECL method. Secondary antibody for these Westerns was at a titer of 1:100000, and images were acquired directly from the membrane using a Fuji LAS-3000 charged-coupled device camera. For co-immunoprecipitation studies, 25 μg of solubilized protein was utilized for 10% SDS−PAGE (with β-mercaptoethanol) and Western blots were carried out as described above except with the standard ECL reagents. The remaining solubilized protein (~150 μg) was incubated for 18 h at 4 °C with 30 μL of precleared anti-Flag M2 agarose beads (Sigma). Subsequently, beads were washed three times by centrifugation and resuspension in cold lysis buffer, then resuspended in 40 μL of 2× SDS sample buffer, boiled for 5 min, and centrifuged, and the proteins were fractionated on 10% SDS−polyacrylamide gels. Western blots were performed using a 1:500 dilution of the HA antibody and the standard ECL detection kit and X-ray film.

Intact Cell Receptor Phosphorylation. Cells coexpressing various receptor constructs and GRK2 were subjected to whole cell receptor phosphorylation studies essentially as described previously (*10*, *25*). Briefly, transfected cells were grown in 100 mm plates and incubated in 3 mL of serum-free and phosphate-free medium containing [^{32}P]orthophosphate (500 μCi/mL) for 2 h at 37 °C in a 5% CO_2 atmosphere. Cells were then incubated in the presence or absence of 10 μM norepinephrine for 15 min, washed five times with ice-cold PBS, and solubilized by rotation in a microcentrifuge tube for 2 h at 4 °C in solubilization buffer consisting of 1% Triton X-100, 0.05% SDS, 0.5 mM EGTA, 1 mM EDTA (pH 7.40), 10 mM NaF, and 10 mM sodium pyrophosphate, in PBS, with the aforementioned protease inhibitors. Unsolubilized material was removed by centrifugation at 20000*g* at 4 °C for 10 min. HA-tagged receptors were immunoprecipitated by incubating the supernatant with protein G−agarose beads and a 1:200 dilution of the anti-HA antibody for 18 h at 4 °C. Following immunoprecipitation, the beads were washed three times by centrifugation and resuspension in cold solubilization buffer. After the final wash, beads were resuspended in 2× SDS sample buffer, boiled for 5 min, and centrifuged, and proteins in the supernatant were fractionated on a 10% SDS−polyacrylamide gel. Signals were acquired with a Molecular Dynamics phosphorimager.

Confocal Microscopy. To localize α_2AR−YFP and β-arrestin−GFP proteins, live cell fluorescent microscopy was performed using an inverted Zeiss LSM 510 laser scanning confocal microscope as previously described (*23*). Transfected cells were transferred to collagen-coated glass bottom dishes, and 48 h post-transfection, cells were treated with the indicated concentration of agonist and real time confocal microscopy was carried out at 37 °C using excitation at 488 nm and a 515−540 nm emission filter.

Miscellaneous. Radioligand binding was performed with [^{3}H]yohimbine on cell membrane fractions as described previously (*7*). Protein concentrations were determined by the copper bicinchoninic acid method (*26*). Studies of p44/p42 MAP kinase and Akt activation by the α_2AR agonist UK14304 were carried out as described previously (*27*, *28*) using monoclonal antibodies which recognize the phosphorylated forms (Cell Signaling Technologies). BRET and radioligand binding data were fit to nonlinear regression functions using Prism (GraphPad). Phosphorylation data from the phosphorimager output were quantitated using ImageQuant (Molecular Dynamics). Results were compared by paired or unpaired *t*-tests, with significance imparted at $P < 0.05$. Data are presented as means ± standard errors.

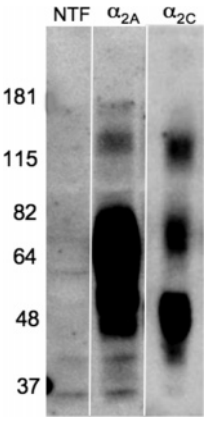

FIGURE 1: Identification of potential α_2AR oligomers by SDS−PAGE and Western blots. HEK-293 cells were transfected with HA-tagged constructs, and SDS−PAGE was carried out under nonreducing conditions. Western blots were performed using the HA antibody and a highly sensitive ECL detection system (see Experimental Procedures). NTF means nontransfected.

RESULTS AND DISCUSSION

Western Blots of Protein from Transfected Cells Reveal Potential Oligomeric α_{2A} and α_{2C}AR. Oligomers of 7-TM receptors show variable extents of stability under conditions of SDS−PAGE. As a screen for potential dimers or higher-order oligomers, HEK-293 cells were transfected with HA-α_{2A}, HA-α_{2C}, or both, and solubilized membranes were subjected to SDS−PAGE under nonreducing conditions followed by Western blots using the HA antibody. As shown in Figure 1, several receptor-specific immunoreactive bands were identified for each receptor. For the α_{2A}AR, the nonglycosylated form (46 kDa), two glycosylated monomers with broad bands centered at 55 and 68 kDa, a potential dimer at approximately twice the molecular mass (~140 kDa) of the latter monomer, and a potential higher-order oligomer at ~165 kDa (~3-fold greater than the 55 kDa form) were detected. For the α_{2C}AR, three forms were detected: a broad band centered at ~48 kDa representing the minimally glycosylated monomer, a higher broad band centered at ~70 kDa, and a potential dimer with a band that

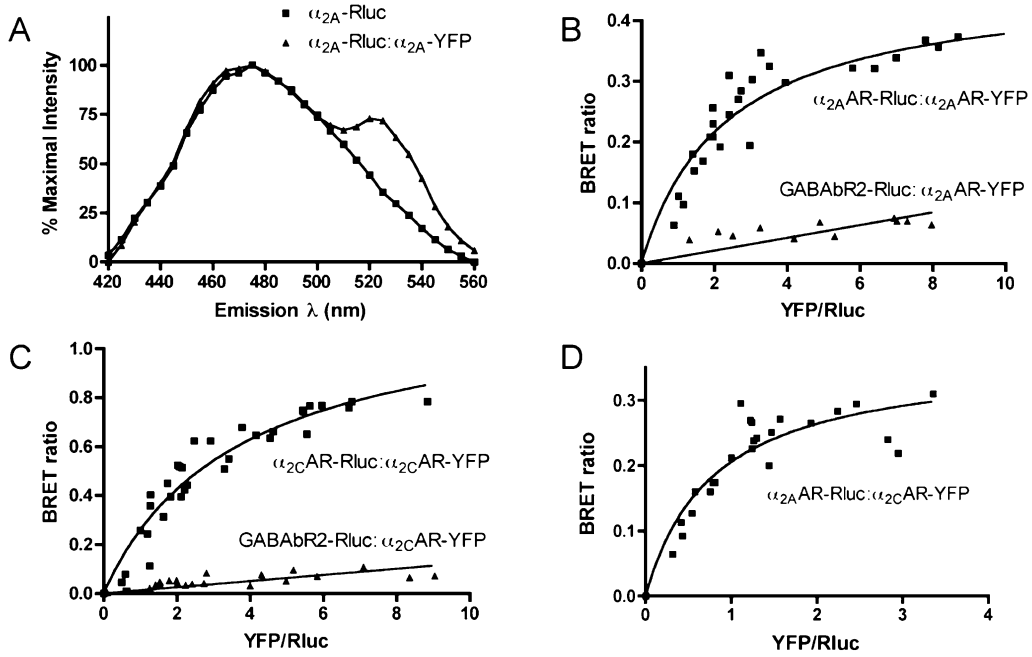

FIGURE 2: BRET indicates α_{2A}AR and α_{2C}AR homo- and heterodimerization. (A) Shown is a representative experiment of the emission spectra of cells expressing α_{2A}−Rluc only and α_{2A}−Rluc with α_{2A}−YFP. The second emission peak at 530 nm is indicative of excitation of α_{2A}−YFP by α_{2A}−Rluc within the dimer. (B and C) Saturation of BRET ratios for α_{2A} and α_{2C} homodimers, respectively, plotted as a function of increasing energy acceptor concentration (fluorescence emission of YFP/light emission of Rluc). In contrast, signals for GABAb R2−Rluc coexpressed with α_{2A}−YFP, and GABAb R2−Rluc coexpressed with α_{2C}−YFP, were linear and did not saturate. (D) BRET ratios for coexpressed α_{2A}−Rluc and α_{2C}−YFP saturate with increasing YFP:Rluc ratios. Each curve represents a composite of five individual experiments, and each point is the mean of three replicates. Curves were compared by analysis of covariance. See the text for results.

ranged from ~120 to 140 kDa. Proteins from nontransfected cells revealed no specific immunoreactive bands. While it appears that the oligomeric forms (particularly for the α_{2A}AR) represent the minority of the total receptors, such quantitation of immunoreactive bands does not readily define this proportion since oligomers are variably stable to the conditions of SDS−PAGE (*29*).

BRET Indicates α_{2A}- and α_{2C}AR Homo- and Heterodimerization. To rigorously ascertain whether the α_{2A}- and α_{2C}ARs form oligomers (for simplicity heretofore termed homo- or heterodimers), BRET was carried out using receptors with in-frame fusions at their carboxy termini with either Rluc or YFP. When these two moieties are close to each other, energy transfer between the donor molecule (Rluc) and the acceptor molecule (YFP) occurs. BRET ratios can subsequently be determined by calculating the ratio of energy emitted by the receptor−YFP construct over that emitted by the receptor−Rluc construct at the emission wavelength for YFP (see Experimental Procedures). The results from BRET studies are shown in Figure 2A−D. A representative emission spectrum for the α_{2A}−Rluc construct expressed alone, and in combination with the α_{2A}−YFP construct, is depicted in Figure 2A. After coelenterazine h exposure, the α_{2A}−Rluc construct alone revealed an emission with a single maximum at 475 nm; upon coexpression of the α_{2A}−YFP construct, a second peak at 530 nm was observed, indicating the proximity and potential dimer formation. In additional studies, with varying amounts of receptor−YFP construct and increasing YFP:Rluc ratios, the BRET signals for both α_{2A}- and α_{2C}AR homodimers reached saturation (Figure 2B,C). Such saturation of the signals indicated that the interaction between receptor pairs was consistent with homodimerization and unlikely to represent random collisions due to overexpression ("pseudo-BRET" signals). This was further examined in experiments in which the GABAb R2 receptor, which is highly divergent in its amino acid composition, was considered to be unlikely to form dimers with α_{2A}- or α_{2C}ARs. In cells expressing GABAb R2-Rluc and α_{2A}−YFP construct, only low-level signals were observed which increased in a linear fashion without reaching saturation (Figure 2B). Similar results were found for cells coexpressing GABAb R2 and α_{2C}−YFP construct (Figure 2C). These data further confirmed that the signals from the BRET experiments represent α_{2A}−α_{2A} and α_{2C}−α_{2C} homodimers.

$BRET_{max}$ derived from saturation curves for α_{2A}AR and α_{2C}AR homodimers were 0.47 ± 0.035 and 1.21 ± 0.011, respectively ($P = 0.02$). The amplitude of the BRET ratio is in part a function of the distance between the energy donor and acceptor. Thus, the higher $BRET_{max}$ value observed for α_{2C}AR dimers is most readily interpreted as a conformation that allows the Rluc and YFP moieties, localized to the C-termini of each fusion receptor, to be closer to each other in this homodimer, compared to an α_{2A}AR homodimer. However, this cannot be interpreted as a relative measure of the distance between the "bodies" of the two receptors, or the propensity to form the oligomer. The relative "affinity" of α_{2A}- and α_{2C}AR to form homodimers, though, can be considered the YFP:Rluc expression ratio required to reach half of the maximum BRET signal, termed $BRET_{50}$. Here, $BRET_{50}$ values were not different for α_{2A}AR homodimers and α_{2C}AR homodimers (2.31 ± 0.44 and 3.67 ± 0.69, respectively). Potential heterodimer formation was assessed in a similar manner by cotransfecting both subtypes (Figure 1D). The $BRET_{max}$ for the α_{2A}−α_{2C} heterodimer was 0.37 ± 0.034. Interestingly, the $BRET_{50}$ of the heterodimer, 0.79 ± 0.2, was lower than that of α_{2A}AR and α_{2C}AR homodimers ($P < 0.01$). Thus, there appears to be enhanced affinity for

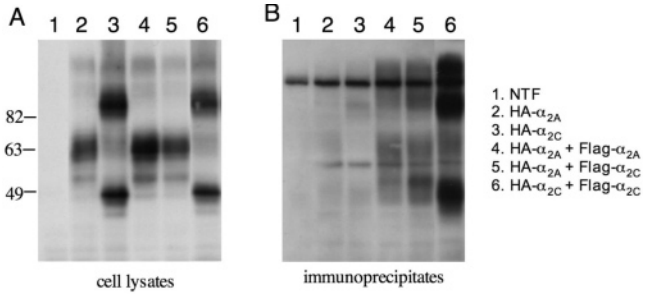

FIGURE 3: Co-immunoprecipitation of dimeric α_2ARs. Western blots using anti-HA antibody of whole cell lysates (A) and anti-FLAG immunoprecipitates (B) prepared from transiently transfected HEK-293 cells. Cells were transfected with HA-α_{2A} only (lane 2), HA-α_{2C} only (lane 3), HA-α_{2A} with Flag-α_{2A} (lane 4), HA-α_{2A} with Flag-α_{2C} (lane 5), and HA-α_{2C} with Flag-α_{2C} (lane 6). In panel A, Western blots were performed using the HA antibody on cell lysates; in panel B, Western blots were performed with the HA antibody on proteins immunoprecipitated with the FLAG antibody. Lane 1 contained protein from nontransfected (NTF) cells. Shown are representative blots from three or four experiments that were performed.

$\alpha_{2A}-\alpha_{2C}$ heterodimers versus $\alpha_{2A}-\alpha_{2A}$ or $\alpha_{2C}-\alpha_{2C}$ homodimers, suggesting that when α_{2A}- and α_{2C}AR are coexpressed, the heterodimerized state may be favored over the respective homodimers. Concomitant radioligand binding studies showed that BRET signals were detectable, particularly for the heterodimer, at expression levels as low as ~500 fmol/mg.

Co-Immunoprecipitation Confirms α_{2A}- and α_{2C}AR Homo- and Heterodimerization. These experiments were performed using differentially tagged receptors (HA-α_{2A}, HA-α_{2C}, Flag-α_{2A}, and Flag-α_{2C}) expressed in various combinations in HEK-293 cells. From the same transfection, Western blots were performed in parallel on cell lysates and immunoprecipitated proteins. The immunoprecipitation of whole cell lysates was performed using an anti-Flag antibody followed by Western blots using an anti-HA antibody. These blots were not optimized for the higher-molecular mass species. Western blots (Figure 3A) with HA antibody of lysates from cells individually expressing the α_2ARs revealed HA-α_{2A} as a band migrating at ~65 kDa (lanes 2, 4, and 5) and HA-α_{2C} as two bands migrating at ~95 and ~40 kDa (lanes 3 and 6). Following transfections of the indicated constructs, immunoprecipitation with FLAG antibody and Western blotting with HA antibody (Figure 3B) revealed immunoreactivity of HA-α_{2A} and HA-α_{2C} (lanes 4 and 6, respectively), indicating a homodimerized state. And, when the two receptors were cotransfected, heterodimerization was indicated by appropriate immunoreactive bands shown in lane 5. Of note, no HA immunoreactivity was observed with FLAG immunoprecipitates prepared from nontransfected cells (lane 1) or with cells expressing HA-α_{2A} and HA-α_{2C} alone (lanes 2 and 3, respectively). Similar to the Western blots of whole cell lysates, co-immunoprecipitated HA-α_{2A} migrated at ~65 kDa and HA-α_{2C} migrated at ~95 and ~45 kDa. For co-immunoprecipitated HA-α_{2A}, additional bands were seen also at ~100 and ~45 kDa. Taken together, these results indicate a physical association consistent with homodimerization of α_{2A}AR and α_{2C}AR and heterodimerization of the two subtypes. These results are consistent with the BRET data, which also indicated homo- and heterodimer formation.

Heterodimerization of α_{2C}AR and α_{2A}AR Does Not Alter the Cellular Distribution of Receptors. Given that α_{2C}AR are known to have a prominent intracellular localization, we considered the possibility that α_{2C}AR in the context of the heterodimer with the predominantly cell surface-expressed α_{2A}AR may have a different distribution. To address this, cells were transfected with the α_{2A}AR−YFP construct alone to visualize the receptor distribution without α_{2C}AR, and then the α_{2A}AR−YFP construct and untagged α_{2C}AR were transfected, the latter not being visualized but nevertheless forming the heterodimer with the α_{2A}AR−YFP construct. Similar transfections were performed with the α_{2C}AR−YFP construct, and the α_{2C}AR−YFP construct with untagged α_{2A}AR. As shown in panels A and C of Figure 4, the α_{2A}AR−YFP construct was predominantly expressed on the cell surface, while both cell surface and a significant intracellular localization was noted for the α_{2C}AR−YFP construct. Cotransfection with untagged receptor subtypes did not alter this distribution pattern (Figure 4B,D). So unlike what has been observed for the α_{1B} and α_{1D} receptors (*30*), the α_{2A}AR does not appear to act as a "chaperone" to enhance cell surface expression of the α_{2C}AR. Nor does α_{2C}AR retard cell surface expression of the α_{2A}AR.

Consequences of the $\alpha_{2A}-\alpha_{2C}$ Heterodimer. An early event in α_{2A}AR signaling is agonist-promoted phosphorylation of the receptor by GRKs (*4*, *10*). Given that such phosphorylation does not occur with the α_{2C}AR (*4*, *10*), we initially concentrated on β-arrestin recruitment and receptor phosphorylation in whole cells when considering the consequences of formation of $\alpha_{2A}-\alpha_{2C}$ heterodimers. To examine β-arrestin recruitment, cells were transfected with a β-arrestin2−GFP fusion construct, along with HA-α_{2A}, HA-α_{2C}, or both. In these experiments (Figure 5), the cellular distribution of the β-arrestin2−GFP fusion was monitored in live cells before and after addition of 10 μM norepinephrine. As shown in panels A, C, and E, prior to the addition of agonist, the β-arrestin2−GFP fusion was evenly distributed throughout the cytoplasm of transfected cells. In α_{2A}AR-expressing cells, the β-arrestin2−GFP fusion was rapidly (maximal response within 5 min) redistributed, as indicated by a marked decrease in cytosolic signal and punctate accumulation at the plasma membrane (panel B). In contrast, no β-arrestin2−GFP redistribution was observed following addition of agonist in cells expressing α_{2C}AR (panel D). Norepinephrine treatment of α_{2A}- and α_{2C}AR-cotransfected cells evoked little β-arrestin2 redistribution (panel F).

These qualitative studies suggested that the α_{2C}AR component of the heterodimer may decrease the level of α_{2A}AR phosphorylation by GRKs. To approach this quantitatively, whole-cell receptor phosphorylation studies of cells expressing GRK2 and HA-α_{2A}, HA-α_{2C}, or HA-α_{2A} coexpressed with *untagged* α_{2C}AR were carried out, immunoprecipitating the receptors with the HA antibody. In these experiments, receptor expression of the individually expressed HA-tagged receptors was equivalent as determined by Western blots and radioligand binding (see the legend of Figure 6). For the HA-α_{2A}/α_{2C}AR cotransfections, the HA-α_{2A} signal by Western blot was equivalent to that of the single transfected HA-α_{2A}, and the total level of α_2AR expression (representing both subtypes) as determined by [^{3}H]yohimbine binding was at least 2-fold greater than that in cells from either of the single transfections. The level of agonist-promoted phos-

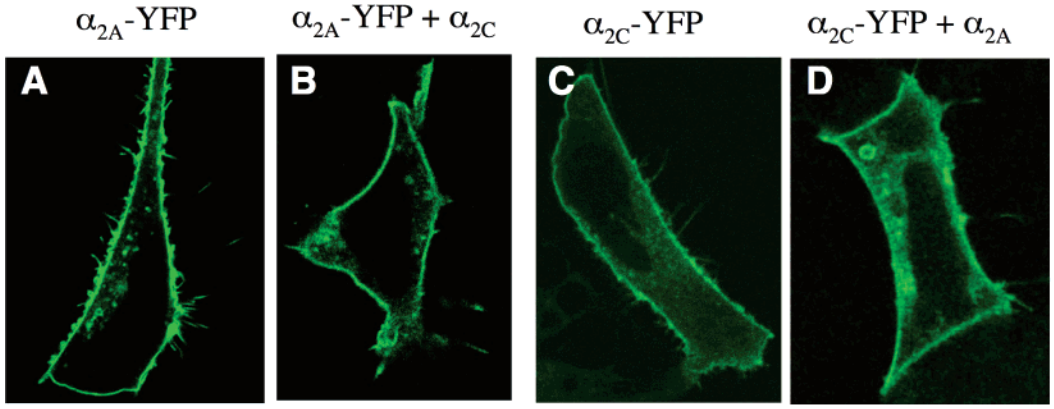

FIGURE 4: Cellular distribution of α_{2A}AR, α_{2C}AR, and the $\alpha_{2A}-\alpha_{2C}$ heterodimer. Shown are representative confocal images from live cells expressing the indicated receptors. α_{2A}AR−YFP is expressed almost exclusively on the cell surface (A) even when cotransfected with untagged α_{2C}AR (B). α_{2C}AR−YFP is expressed on the cell surface, as well as intracellularly (C). This phenotype is unaltered by coexpression of untagged α_{2A}AR (D). Shown are representative results from three to five experiments with 10−20 images acquired from each experiment.

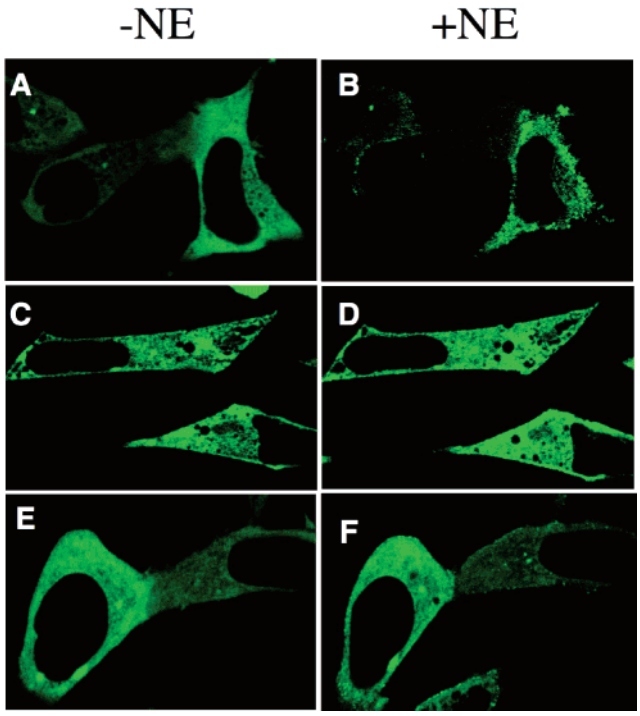

FIGURE 5: Agonist-promoted α_{2A}AR-mediated β-arrestin recruitment is impaired when in the context of the $\alpha_{2A}-\alpha_{2C}$ heterodimer. HEK-293 cells were transfected with plasmids to coexpress β-arrestin2−GFP with either α_{2A}AR (A and B), α_{2C}AR (C and D), or both subtypes (E and F). Confocal microscopy of live cells was carried out as described in Experimental Procedures. In the absence of agonist (A, C, and E), β-arrestin2 is distributed homogeneously throughout the cytoplasm. After agonist exposure for 5 min, the α_{2A}AR-expressing cells show a redistribution of β-arrestin2, indicated by a decrease in the magnitude of the cytosolic signal and punctate accumulation at the cell surface. Agonist exposure failed to promote such recruitment in cells expressing α_{2C}AR (D) or the $\alpha_{2A}-\alpha_{2c}$ heterodimer (F). Shown are representative images from 10 experiments, with 10−20 cells imaged per experiment.

phorylation of the α_{2A}AR, which was >10-fold greater than basal, is shown in Figure 6A (lanes 1 and 2). The level of agonist-promoted α_{2C}AR phosphorylation was not statistically greater than that of basal (Figure 4, lanes 3 and 4). The $\alpha_{2A}-\alpha_{2C}$ heterodimer (lanes 5 and 6) underwent agonist-promoted phosphorylation amounting to 55 ± 3.4% of the α_{2A}AR homodimer ($n = 4$, $P = 0.01$; Figure 6A, lanes 7 and 8, and Figure 6B). These results indicated that the conformation of the α_{2A}AR when in the $\alpha_{2A}-\alpha_{2C}$ heterodimer represents a less favorable substrate for agonist-promoted GRK2-mediated phosphorylation as compared to the α_{2A} homodimer. This phenotype could not be related to an altered affinity of the heterodimer for the agonist NE, as the "composite" affinities (representing a combination of the two homodimers and the heterodimer) when both receptors were expressed were comparable to those of α_{2A}- or α_{2C}AR-expressing cells (data not shown). Recent studies have implicated β-arrestin signaling as a component of 7-TM receptor-mediated activation of p44/p42 MAP kinase (*31*) and Akt (*28*, *32*). The phenotype of the two early events (attenuated GRK phosphorylation and β-arrestin recruitment) observed with the $\alpha_{2A}-\alpha_{2C}$ coexpressing cells suggested that one or both of these downstream signals might also be depressed with the heterodimer. We found that agonist-promoted Akt activation was depressed in the α_{2A}- and α_{2C}AR-coexpressing cells compared to that in α_{2A}AR-expressing cells (Figure 7). This was despite the level of α_{2A}AR expression being equivalent, or higher, in the cotransfected cells compared to the α_{2A}AR-only-expressing cells. In contrast, p44/p42 MAP kinase activation was not different between the two in terms of the maximal stimulation or the EC_{50} (data not shown).

Taken together, these data indicate that in cells only expressing α_{2A} or α_{2C}AR, these receptors can both exist as dimers. The functional significance of the dimer, as compared to an α_{2A}- or α_{2C}AR that is not in the dimerized state, remains unclear. And, as for many 7-TM receptors, such relevance is difficult to ascertain since any mutation that disrupts oligomerization may also alter receptor signaling or ligand binding, thereby making it difficult to ascertain the phenotype of a nondimerized receptor. However, we show that α_{2A}- and α_{2C}AR form heterodimers and define the properties of this unit compared to the individual homodimers. On the basis of the BRET data, it appears that in cells expressing both subtypes, the $\alpha_{2A}-\alpha_{2C}$ heterodimer may be preferred over the homodimers. This is particularly relevant in sympathetic neurons, where the distal presynaptic terminal expresses α_{2A}AR and α_{2C}AR (*6*). As introduced earlier, the incongruities between physiologic studies and the pharmacology of the receptors individually expressed in cells suggested to us that a heterodimer could be the functional "α_2AR" unit. We show three events that occur after binding

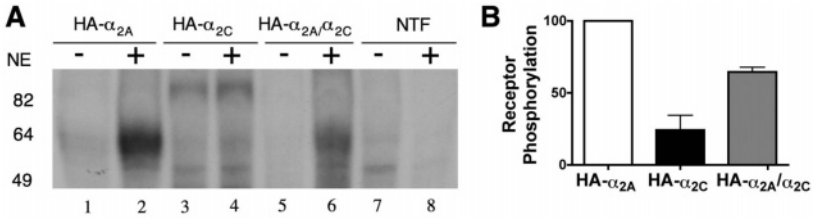

FIGURE 6: α_{2C}AR heterodimerization with α_{2A}AR decreases the level of α_{2A}AR phosphorylation by GRK2. Cells coexpressing equivalent levels of HA-tagged receptor, HA-α_{2A}AR, HA-α_{2C}AR, and HA-α_{2A}AR in the presence of untagged α_{2C}AR, were incubated with [^{32}P]orthophosphate, exposed to 10 μM norepinephrine for 15 min, and purified by immunoprecipitation with the HA antibody as described in Experimental Procedures. An autoradiogram from a representative experiment is shown in panel A. Gels were quantitated using a Molecular Dynamics phosphorimager. For the experiments that are depicted, expression levels were as follows: 3120 fmol/mg for HA-α_{2A}AR-only transfected cells, 4070 fmol/mg for HA-α_{2C}AR-only cells, and 9070 fmol/mg for HA-α_{2A}AR with α_{2C}AR co-expressing cells. Mean data from four experiments are shown in panel B. NE represents norepinephrine.

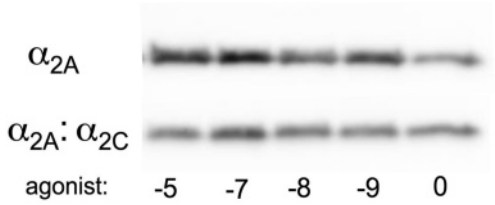

FIGURE 7: Heterodimer formation alters agonist-promoted activation of Akt. HEK-293 cells were transfected to express α_{2A}AR, or α_{2A}AR and α_{2C}AR, and treated for 5 min with the indicated concentrations (log[M]) of the α_2AR agonist UK14304 at 37 °C. Solubilized cell lysates (20 μg/sample) were subjected to SDS−PAGE and Western blots carried out with antibody recognizing the phosphorylated form of Akt. In the representative experiment shown, the level of α_{2A}AR expression was 481 fmol/mg in the singly transfected cells, and the combined α_{2A}AR and α_{2C}AR cells expressed 933 fmol/mg (with equal expression of both subtypes). In six such experiments, the maximal phosphorylation of Akt from $\alpha_{2A}-\alpha_{2C}$ heterodimer-expressing cells was 71 ± 5% of that from α_{2A}AR-expressing cells ($P < 0.01$).

of agonist to the α_{2A}AR, GRK-mediated phosphorylation, β-arrestin recruitment, and Akt activation, are inhibited when α_{2A}AR is in the context of the heterodimer as opposed to the α_{2A}AR homodimer.

While a number of functional consequences of 7-TM receptor heterodimerization have been ascertained (reviewed in refs *33* and *34*), the current results with α_{2A}- and α_{2C}AR are unique, although the characteristics of the $\alpha_{2A}-\alpha_{2C}$ heterodimer are somewhat similar to recently published studies of a βAR heterodimer composed of β_2 and β_3 (*24*). The main findings from this work were that the heterodimer has a lower level of β-arrestin recruitment compared to β_2AR, and it displayed a loss of agonist-promoted internalization compared to β_2AR. In addition, the heterodimer failed to stimulate p44/p42 MAP kinase, in contrast to β_2AR and β_3AR expressed separately. Agonist-promoted phosphorylation or baseline cellular distribution of receptors was not assessed. For the $\alpha_{2A}-\alpha_{2C}$ heterodimer, we also note a loss of agonist-promoted β-arrestin recruitment. We explored this mechanistically and found a significant decrease in the level of GRK-mediated phosphorylation of α_{2A}AR in the heterodimer compared to that in α_{2A}AR expressed alone. Given the link between receptor phosphorylation by GRKs and β-arrestin recruitment, we contend that the mechanism of altered β-arrestin recruitment observed with the heterodimer is due to this loss of phosphorylation. Since the potential phosphorylation sites for α_{2A}AR in the third intracellular loop are still present in the heterodimer, these results indicate a conformational change in the receptor, potentially involving the transmembrane domains or the intracellular loops, which place the α_{2A}AR in an unfavorable conformation for GRK-mediated phosphorylation as compared to the homodimer. Interestingly, studies with third-loop fusion proteins indicate that β-arrestin2 can bind in vitro to the third loop of the α_{2C}AR but not the α_{2A}AR (*35*). These types of studies, however, do not address agonist effects which would be operative in whole cells during receptor activation. Nevertheless, there is some consistency with the aforementioned in vitro studies and those assessing agonist-promoted internalization of the α_2AR subtypes. β-Arrestin2 overexpression increased the degree of internalization by ~1.5-fold after agonist exposure for 20 min for both subtypes (*8*). However, the absolute magnitude of internalization is greater for α_{2C}AR than α_{2A}AR [~35 and 15%, respectively (*8*)]. In our current studies of the heterodimer, we did not feel that this phenotype could be distinguished given this relatively small difference and the fact that homodimers and heterodimers are present in coexpressing cells. Nevertheless, in our β-arrestin recruitment studies, we rarely found agonist-promoted recruitment in $\alpha_{2A}-\alpha_{2C}$ transfected cells, despite the fact that α_{2A}AR was expressed at levels equivalent to that of control (α_{2A}AR alone) cells.

We recognize that differences in receptor phenotypes established in the current work, which utilized HEK-293 cells, are early events ascertained in a reductionist model system. The long-term, multistep, downstream consequences of altered GRK phosphorylation or β-arrestin signaling are best ascertained in endogenously expressing cells in the proper physiologic milieu. Such studies could utilize in vivo studies of the α_2AR knockout mice and highly selective, subtype-specific, agonists (when they become available). Interestingly, the current work may impact the development of such agents for therapeutic purposes. There is considerable interest in agonists which act specifically at the α_{2A}AR or α_{2C}AR. As discussed earlier, on the basis of previous work by our group (*2*) and others (*4*), certain assumptions might be made about the potential for tachyphylaxis to such drugs. Our current findings suggest that these early paradigms need to be reconsidered, when the cell type of interest for targeting pharmacologic therapy expresses both the α_{2A}- and α_{2C}AR subtypes. In addition, the existence and functional state of the heterodimer reveal a unique phenotype compared to that described for other 7-TM receptors and further show the capacity of receptors to diversify function by forming oligomers with other receptors. This property of the α_2ARs may shed additional light on how endogenous agonists evoke

specialized physiologic responses which depend on whether there is expression of only one or two α_2AR subtypes.

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