

α_{2C} -Adrenergic Receptors Exhibit Enhanced Surface Expression and Signaling upon Association with β_2 -Adrenergic Receptors

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ABSTRACT

The α_{2C} -adrenergic receptor (α_{2C} AR) is known to be poorly trafficked to the cell surface when expressed in a variety of cell types. We tested the hypothesis that the surface expression and signaling of α_{2C} AR might be enhanced by heterodimerization with other G protein-coupled receptors (GPCRs). Cotransfection of α_{2C} AR with more than 25 related GPCRs revealed that only coexpression with the β_2 -adrenergic receptor (β_2 AR) increased the surface localization of α_{2C} AR in human embryonic kidney-293 cells. Coimmunoprecipitation of α_{2C} AR with β_2 AR confirmed a physical interaction between the two receptors. Confocal microscopy studies demonstrated that α_{2C} AR expressed alone was mainly intracellular, whereas α_{2C} AR coexpressed with β_2 AR was predominantly localized to the plasma membrane. Ligand binding studies revealed a signifi-

cant increase in α_{2C} AR binding sites upon coexpression with β_2 AR, with no apparent change in affinity for α_2 AR ligands. Functional assays with the α_2 AR-specific agonist brimonidine (UK 14,304) revealed that coexpression of β_2 AR with α_{2C} AR enhanced α_{2C} AR-mediated activation of extracellular signal-regulated kinase 1/2. Furthermore, analyses of agonist-promoted receptor endocytosis demonstrated enhanced α_{2C} AR internalization in response to α_2 AR agonists when α_{2C} AR and β_2 AR were coexpressed. In addition, substantial cointernalization of α_{2C} AR in response to β AR agonists was observed when α_{2C} AR was coexpressed with β_2 AR. These data reveal that α_{2C} AR can interact with β_2 AR in cells in a manner that regulates α_{2C} AR surface expression, internalization, and functionality.

The adrenergic receptors are a family of cell-surface G protein-coupled receptors (GPCRs) that mediate the actions of the hormone epinephrine and the neurotransmitter norepinephrine. The three main adrenergic receptor (AR) classes (α_1 , α_2 , and β_2) can be further divided into three subtypes each, and all of these subtypes are excellent targets for therapeutic pharmaceuticals. The specific roles of the various adrenergic receptor subtypes is becoming increasingly clear through studies on knock-out mice (Philipp and Hein, 2004), and novel therapies making use of these insights await the development of more subtype-specific drugs. However, two of the adrenergic receptor subtypes, α_{2C} AR and α_{1D} AR, have proven extremely difficult to study in heterologous expression systems, because they do not traffic efficiently to the cell

surface when expressed alone and are therefore largely non-functional (von Zastrow et al., 1993; Daunt et al., 1997; Chalothorn et al., 2002). Recently, it has been shown that α_{1D} AR surface expression and functionality can be profoundly enhanced by coexpression with α_{1B} AR or β_2 AR, presumably due to receptor heterodimerization (Uberti et al., 2003, 2005; Hague et al., 2004b).

The mechanisms underlying the α_{2C} AR-trafficking defect remain enigmatic and are important to address because of the therapeutic importance of drugs targeting α_2 receptors. It has been shown that α_{2C} AR does traffic efficiently to the cell surface when expressed in several neuronally derived cell types, suggesting that the poor trafficking of α_{2C} AR seen in other cell types is highly dependent on cellular context (Hurt et al., 2000). Other studies suggest that surface expression of α_{2C} AR can be increased by exposure to cold temperatures, which may further contribute to tissue-specific regulation of α_{2C} AR activity (Jeyaraj et al., 2001; Bailey et al., 2004). Studies on α_{2C} AR knockout mice reveal a key role for this subtype in mediating spinal analgesia (Fairbanks et al., 2002) and in the regulation of epinephrine release (Hein et

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic receptor; GABA_BR, GABA_B receptor; DHA, dihydroalprenolol; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; RX 821002, 2-methoxydiazoxan; UK 14,304, brimonidine.

al., 1999; Brede et al., 2003), demonstrating that α_2C AR is functional and relevant *in vivo*. Thus, it seems likely that efficient trafficking of α_2C AR to the cell surface may require an associated partner that is expressed in a cell type-dependent manner. Such a partner could be a specialized chaperone protein, or it could be another receptor.

Classically, GPCRs have been thought to act as monomers. However, a growing body of literature suggests that dimerization is important for the function of many GPCRs. Interestingly, dimerization does not seem to be limited to homodimers, because heterodimerization of GPCRs has been shown to occur as well (Terrillon and Bouvier, 2004; Prinster et al., 2005). Depending on the number of GPCR heterodimers and their functional consequences, the physiological effects mediated by GPCRs may be much larger than could be ascribed to the approximately 750 GPCRs predicted to be contained in the human genome. The possibility of such an increase in receptor variation and a concomitant increase in potential drug targets makes investigation into the functions of GPCR heterodimers an important research direction. Heterodimerization has also been observed among adrenergic receptor subtypes, with various effects described on receptor trafficking and signaling, depending on the receptors involved (Lavoie et al., 2002; Stanasila et al., 2003; Xu et al., 2003; Breit et al., 2004; Hague et al., 2004a, 2006; Uberti et al., 2005). In this study, we investigated whether coexpression with other GPCRs might enhance the surface expression and functionality of α_2C AR.

Materials and Methods

Receptor Constructs. GABA_BR2 was kindly provided by Fiona Marshall (GlaxoSmithKline, Uxbridge, Middlesex, UK). β_1 - and β_2 -Adrenergic receptor constructs were kindly provided by Robert Lefkowitz (Duke University Medical Center, Durham, NC). α_{1A} -, α_{1B} -, and α_{1D} -Adrenergic receptor constructs were kindly provided by Ken Minneman (Emory University School of Medicine, Atlanta, GA). α_{2A} -, α_{2B} -, and α_2C -Adrenergic receptor constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center, Nashville, TN). The β_3 -adrenergic receptor was kindly provided by Sheila Collins (CIIT Centers for Health, Research Triangle Park, NC). The serotonin 5HT_{1A} receptor construct was kindly provided by John Raymond (Medical University of South Carolina, Charleston, SC). Angiotensin AT1 and AT2 receptor constructs, trace amine receptors constructs (1–5), P2Y₂ receptor construct, NPY1 receptor construct, and thromboxane A₂ receptor construct were purchased from the University of Missouri-Rolla cDNA Resource Center (Rolla, MO). Muscarinic m1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). The purinergic receptor P2Y₁ construct was kindly provided by Ken Harden (University of North Carolina, Chapel Hill, NC). Opioid receptor constructs (μ , δ , and κ) were kindly provided by Ping-Yee Law (University of Minnesota Medical School, Minneapolis, MN). The histamine H3 receptor construct was kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute, San Diego, CA).

The FLAG-tagged α_2C -adrenergic receptor was generated from the HA-tagged α_2C AR construct mentioned above. The α_2C AR coding sequence was amplified via polymerase chain reaction using the primers 5'-GACTCTAGAGCGTCCCCAGCGCTG-3' (5' end, containing the XbaI restriction site) and 5'-GTCGGATCCTCACTGCCTGAAGCC-3' (3' end, containing the BamHI restriction site preceded by a stop codon). After polymerase chain reaction amplification, the receptor and plasmid pDoubleTrouble, containing N-terminal sequential hexahistidine and FLAG epitopes, were digested with XbaI

and BamHI restriction enzymes and ligated with T4 DNA ligase, and the sequence was confirmed by DNA sequencing. All molecular biology reagents were obtained from Promega (Madison, WI).

Cell Culture and Transfection. All tissue culture media and related reagents were purchased from Invitrogen (Carlsbad, CA). 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% CO₂ incubator. To express receptors, 2 μ g of DNA from each construct was mixed with Lipofectamine 2000 (15 μ l; Invitrogen) and added to 5 ml of complete medium in 10-cm tissue culture plates containing cells at ~80 to 90% confluence. After overnight incubation, complete medium was added to the culture dishes, and cells were trypsinized and replated on an appropriately sized dish.

For confocal microscopy, a transfection efficiency of >80% was achieved (by transfection) using the Nucleofector solution and following the protocol supplied by the manufacturer (Amaxa, Gaithersburg, MD). In brief, HEK-293 cells were trypsinized, collected by centrifugation, and resuspended in Nucleofector solution along with 1 μ g of each cDNA. The suspension was then subjected to electroporation in the Nucleofector, complete medium was added, and cells were plated directly onto tissue culture-treated glass slides (BD Biosciences, Bedford, MA) and grown for 18 to 24 h.

Surface Expression Assay. HEK-293 cells stably transfected with α_2C AR were transiently transfected with the appropriate epitope-tagged constructs and plated on poly-D-lysine-coated 35-mm dishes. Cells were washed, fixed, and rinsed. Cells were then incubated in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, and 5% w/v nonfat dry milk, pH 7.5) and incubated with horseradish peroxidase-conjugated anti-FLAG M2 (1:1000) or 12CA5 anti-HA (1:1000) monoclonal antibodies in blocking buffer. Cells were washed with blocking buffer and incubated with SuperSignal ELISA ECL reagent for 15 s before the chemiluminescence of the whole 35-mm plate, which corresponds to the amount of receptor on the cell surface, was quantified in a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). For internalization assays, cells were stimulated with the appropriate agent in Dulbecco's modified Eagle's medium for 30 min at 37°C and then placed on ice and fixed before cell surface measurements were made.

Immunocytochemistry and Laser-Scanning Confocal Microscopy. The nucleofected cells were washed and fixed immediately, or to investigate internalization, cells were treated with bromidide (UK 14,304; 10 μ M) or isoproterenol (10 μ M) for 30 min at 37°C and then placed on ice, washed, and fixed. The cells were then blocked and permeabilized by incubating in blocking buffer (1 \times phosphate-buffered saline, 2% bovine serum albumin, and 0.1% saponin, pH 7.4) and incubated with mouse anti-FLAG antibody (1:1000; Sigma, St. Louis, MO) and rat anti-HA antibody (1:1000; Roche, Indianapolis, IN), washed, and incubated with anti-mouse-conjugated Alexa 488 and anti-rat-conjugated Alexa 546 (Molecular Probes, Eugene, OR). The slides were washed and dehydrated and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Cells were scanned with a LSM 510 laser scanning confocal microscope (Carl Zeiss GmbH, Heidelberg, Germany). For detecting Alexa 488, fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected for 510 to 520 nm. For detecting Texas Red, rhodamine fluorescence was excited using a helium-neon laser at a wavelength of 522 nm.

Western Blotting. Samples in 1 \times sample buffer were centrifuged briefly before loading approximately 20 μ l of the sample. The proteins were resolved by SDS-PAGE on a 4 to 20% Tris-glycine gel and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membranes were incubated for 30 min in Tris-buffered saline with 0.1% Tween 20 plus 5% dry milk and then with the appropriate primary antibody for 1 h. The membranes were washed and incubated with a fluorescent-conjugated secondary antibody for 30 min followed by detection using the Odyssey imaging system (Li-Cor, Lincoln, NE).

Assays of ERK Activation. Cells grown on 12-well dishes were starved in serum-free Dulbecco's modified Eagle's medium overnight and exposed to vehicle in the presence or absence of 10 μ M UK 14,304 for 5 min at 37°C, added directly to the starvation medium. At the end of the stimulation, the medium containing the agent was removed, and 60 μ l of 1 \times sample buffer was added. Samples were sonicated, boiled for 5 min, and centrifuged briefly at 17,000g before loading 20 μ l of each sample. The proteins were resolved by SDS-PAGE as described above, and the proteins were detected using monoclonal anti-phospho-p42/44 and rabbit anti-p42/44 antibodies to blot for phosphorylated and total mitogen-activated peptide, respectively. Fluorescent-conjugated secondary anti-mouse and anti-rabbit were then used for detection by scanning using the Odyssey imaging system, and band density was quantified using Odyssey imaging software (Li-Cor).

Coimmunoprecipitation. Membranes of cells transiently transfected were washed and collected in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing Complete protease inhibitor cocktail (Roche) and incubated for 60 min at 4°C with rotation. Unsolubilized membranes were pelleted, and the supernatant was incubated with anti-FLAG-conjugated agarose beads overnight at 4°C with rotation. The beads were washed in phosphate-buffered saline, and the protein was eluted from the beads in 1 \times sample buffer. Samples were analyzed by Western blotting as described above.

Radioligand Binding Assays. Cells were washed, collected, and centrifuged at 50,000g to collect the membranes, sonicated briefly, and resuspended in 3 ml of fresh binding buffer. The affinity of the receptors for [³H]dihydroalprenolol (DHA) (β_2 AR antagonist) or [³H]rauwolscine (α_2 CAR antagonist) was assessed in saturation binding assays using six concentrations of [³H]DHA or [³H]rauwolscine. The membrane preparation was incubated with [³H]DHA or [³H]rauwolscine for 30 min at 22°C. The reaction was stopped by filtration through Whatman GF/C glass fiber filters (Whatman Schleicher and Schuell, Keene, NH) on a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The amount of ³H ligand present was determined by liquid scintillation counting. Nonspecific binding was defined using 10 mM propranolol for β_2 AR or 10 mM norepinephrine or RX 821002 (2-methoxyidazoxan) for α_2 CAR. Nonlinear regression analyses of saturation binding assays and statistical comparisons were performed with Prism (GraphPad Software, Inc., San Diego, CA).

Results

Localization of α_2 CAR following Cotransfection with Other GPCRs. To investigate the effect of heterodimerization on α_2 CAR surface expression, α_2 CAR was coexpressed with a panel of 29 different GPCRs. The relative increase in FLAG-tagged α_2 CAR surface expression was investigated using an intact-cell ELISA assay that has been used previously to study other trafficking-defective GPCRs (Uberti et al., 2003, 2005; Hague et al., 2004a,b). Coexpression with most of the receptors examined had no detectable effect on the localization of α_2 CAR, but cotransfection with β_2 AR caused a marked increase (4-fold) in the amount of α_2 CAR at the cell surface (Fig. 1).

The effect of β_2 AR on α_2 CAR localization was confirmed via a second and independent technique, confocal microscopy. As described previously (von Zastrow et al., 1993; Daunt et al., 1997), α_2 CAR expressed alone in HEK-293 cells is largely intracellular (Fig. 2A). In contrast, β_2 AR expressed alone in our studies displayed strong surface localization in HEK-293 cells (Fig. 2B). Upon coexpression of β_2 AR with α_2 CAR, β_2 AR localization was unaltered by coexpression with α_2 CAR, but

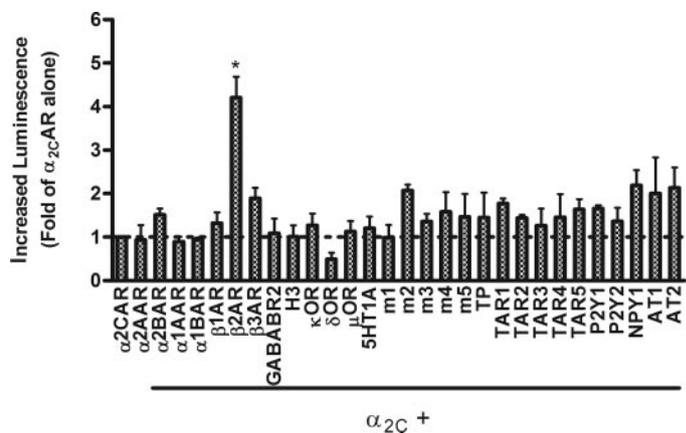


Fig. 1. Coexpression with β_2 AR enhances α_2 CAR surface expression. HEK-293 cells were transfected with α_2 CAR alone or cotransfected with α_2 CAR plus other GPCRs. After 48 h, the cells were fixed, and FLAG-tagged α_2 CAR was labeled with anti-FLAG horseradish peroxidase-conjugated antibody. Relative luminescence was quantified using a luminometer following incubation with ELISA ECL reagent. Where possible, the presence of the cotransfected receptors was confirmed by Western blot. Data shown are from three to six separate experiments for each condition. Receptor abbreviations: H, histamine receptor; OR, opioid receptor; 5HT_{1A}, serotonin receptor 1A; m, muscarinic receptor; TP, thromboxane A2 receptor; P2Y, purinergic receptor; TAR, trace amine receptor; NPY, neuropeptide Y receptor; AT, angiotensin receptor. *, $p < 0.001$.

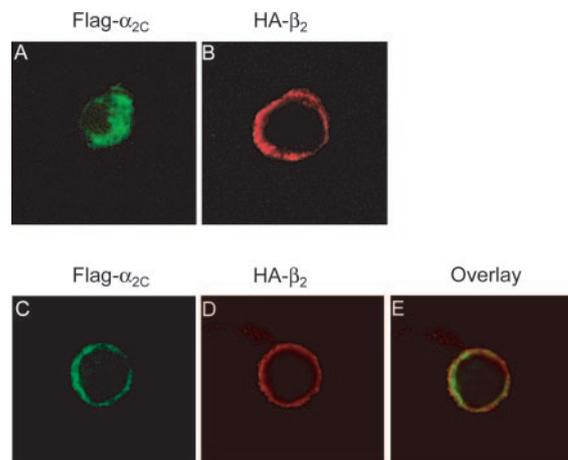


Fig. 2. Coexpression of α_2 CAR with β_2 AR alters the subcellular localization of α_2 CAR. FLAG- α_2 CAR (A, green) and HA- β_2 AR (B, red) were expressed alone or together (C–E) in HEK-293 cells and visualized using secondary antibodies coupled to Alexa 488 or Alexa 546. In the absence of β_2 AR, α_2 CAR was mainly intracellular. However, α_2 CAR was found predominantly at the cell surface following coexpression with β_2 AR (C–E). These data are representative of at least three separate experiments for each condition.

there was a striking increase in the surface localization of α_2 CAR, such that α_2 CAR colocalized well with β_2 AR at the plasma membrane (Fig. 2, C–E).

One possible explanation for the ability of β_2 AR to alter the trafficking of α_2 CAR is an interaction between the two receptors. To explore this possibility, we investigated the ability of α_2 CAR to interact with β_2 AR by coimmunoprecipitation. Immunoreactivity for FLAG- α_2 CAR was evident as a major band at \sim 45 kDa and as a second band at approximately 100 kDa, which may represent receptor multimers not fully resolved on SDS-PAGE. Both α_2 CAR bands were efficiently immunoprecipitated with anti-FLAG antibodies (Fig. 3). The major band of HA- β_2 AR immunoreactivity (\sim 52 kDa) was not immunoprecipitated by anti-FLAG antibodies when

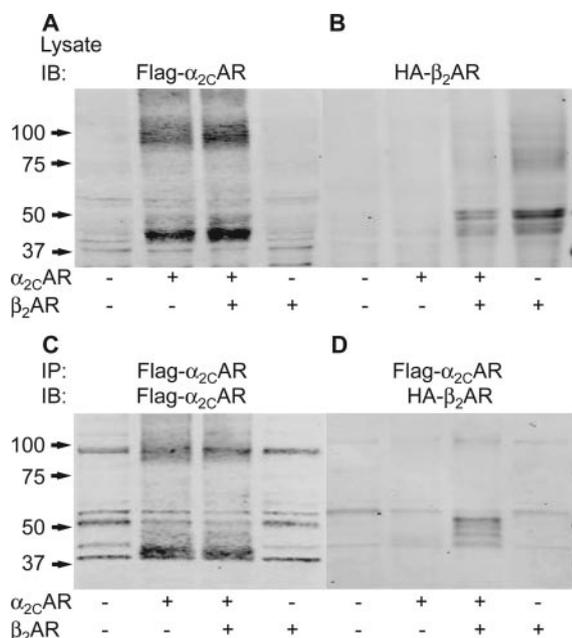


Fig. 3. Coimmunoprecipitation of α_2 C-AR with β_2 -AR. A and B, cells were transfected with FLAG- α_2 C-AR alone FLAG- α_2 C-AR/HA- β_2 -AR, or HA- β_2 -AR alone. The lysates were incubated with anti-FLAG-conjugated beads to immunoprecipitate FLAG- α_2 C-AR. C and D, the immunoprecipitates were examined for FLAG and HA immunoreactivity. HA- β_2 -AR was immunoprecipitated by the anti-FLAG antibodies only when coexpressed with FLAG- α_2 C-AR. Molecular weight standards are indicated by the numbers to the left. This figure is representative of five separate experiments.

β_2 -AR was expressed alone. However, HA- β_2 -AR was robustly coimmunoprecipitated with FLAG- α_2 C-AR when the two receptors were expressed together. These data reveal that α_2 C-AR and β_2 -AR can form a complex in a cellular environment.

Binding Properties of α_2 C-AR and β_2 -AR. The effects of receptor coexpression on binding affinity and total receptor number for α_2 C-AR and β_2 -AR were assessed in saturation binding assays. Using the α_2 AR-specific ligand rauwolscine, we observed that the K_D value was unchanged by coexpression with β_2 -AR but that the B_{max} value was increased by approximately 2-fold. Conversely, neither the K_D nor B_{max} values for [3 H]DHA binding were altered when β_2 -AR was coexpressed with α_2 C-AR (Fig. 4; Table 1).

Because agonist and antagonist binding might plausibly be affected differentially by receptor heterodimerization, we assessed the ability of agonists specific for α_2 C-AR or β_2 -AR to compete with their respective radioligands. However, competition binding assays revealed that the affinity values for UK 14,304 and norepinephrine binding to α_2 C-AR were not significantly different when α_2 C-AR was expressed alone versus coexpressed with β_2 -AR (Fig. 5). The affinity values for epinephrine and isoproterenol binding to β_2 -AR were also not changed when β_2 -AR was coexpressed with α_2 C-AR (Table 1).

Effect of α_2 C-AR/ β_2 -AR Coexpression on α_2 C-AR Functionality. The α_2 AR subfamily is predominantly coupled to $G_{i/o}$ and can strongly activate ERK1/2 (DeGraff et al., 1999). Assays of ERK1/2 phosphorylation are a sensitive, robust method for assessing the signaling activity of many GPCRs. Therefore, we explored the effect of β_2 -AR coexpression on the functional properties of α_2 C-AR using the phosphorylation of ERK1/2 as an endpoint. Cells transfected with α_2 C-AR in the

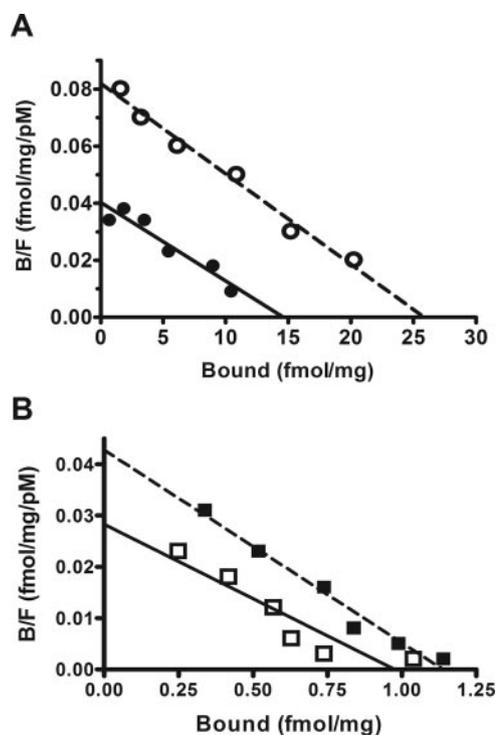


Fig. 4. Coexpression of α_2 C-AR with β_2 -AR increases α_2 C-AR binding sites. Membranes from cells transiently expressing α_2 C-AR, α_2 C-AR/ β_2 -AR, or β_2 -AR were prepared and incubated with varying concentrations of [3 H]rauwolscine (A) or [3 H]DHA (B). The affinity of α_2 C-AR for [3 H]rauwolscine was not altered in the absence (filled circles) or presence (open circles) of β_2 -AR, but the B_{max} was increased (see Table 1). Data shown are representative of three separate experiments. Both the affinity of β_2 -AR for [3 H]DHA and the B_{max} were similar when β_2 -AR was expressed in the absence (filled squares) or presence (open squares) of α_2 C-AR. Data shown are representative of three separate experiments; in all cases, error at each point was less than 15% of the calculated value.

absence or presence of β_2 -AR were incubated with UK 14,304. Increases in ERK1/2 phosphorylation were observed in both cases. However, the observed increase in ERK1/2 phosphorylation was much larger when α_2 C-AR was coexpressed with β_2 -AR than when α_2 C-AR was expressed alone. The stimulatory effects of UK 14,304 in all cases were blocked by RX 821002, an α_2 AR antagonist (Fig. 6). In contrast to the large effect of β_2 -AR coexpression on α_2 C-AR signaling, β_2 -AR-mediated stimulation of ERK1/2 phosphorylation by isoproterenol was not significantly altered by coexpression with α_2 C-AR (data not shown).

The predominantly intracellular localization of α_2 C-AR in most cell types has been a confounding factor in previous studies aimed at assessing the capacity of α_2 C-AR to undergo agonist-promoted endocytosis (Daunt et al., 1997; DeGraff et al., 1999; Olli-Lahdesmaki et al., 1999). However, the ability of β_2 -AR to traffic α_2 C-AR to the plasma membrane enabled us to more easily investigate α_2 C-AR internalization following agonist stimulation. When α_2 C-AR was expressed alone and stimulated with UK 14,304, the small population of α_2 C-ARs on the cell surface did not undergo any significant internalization, as assessed using the luminometer-based whole-cell ELISA assay. When α_2 C-AR was coexpressed with β_2 -AR, however, there was a striking 30% decrease in the amount of α_2 C-AR on the cell surface following a 30-min treatment with UK 14,304. Furthermore, the β_2 -AR-specific agonist isoproterenol also resulted in substantial endocytosis of α_2 C-AR,

TABLE 1

Ligand binding properties of $\alpha_2\text{C}\text{AR}$ and $\beta_2\text{AR}$ expressed separately or in combination

Membranes derived from HEK-293 cells transiently transfected with $\alpha_2\text{C}\text{AR}$ and/or $\beta_2\text{AR}$ were examined in saturation binding assays to determine affinity constants for [^3H]rauwolscine (Rau, α_2 antagonist) or [^3H]DHA ($\beta_2\text{AR}$ antagonist). K_i values for $\alpha_2\text{C}\text{AR}$ agonists (UK 14,304 and norepinephrine) were determined in competition assays with [^3H]Rau, and K_i values for $\beta_2\text{AR}$ agonists (isoproterenol and epinephrine) were determined in competition assays with [^3H]DHA. Values are mean \pm S.E.M. of three to five experiments.

	[^3H]Rau		[^3H]DHA		$\alpha_2\text{AR}$ Agonists		$\beta_2\text{AR}$ Agonists	
	K_D	B_{max} Increase	K_D	B_{max} Increase	UK (K_i)	NE (K_i)	Iso (K_i)	Epi (K_i)
	pM	-Fold	pM	-Fold	μM	μM	μM	μM
$\alpha_2\text{C}\text{AR}$	165 \pm 18	1.83 \pm 0.18	N.D.	N.D.	0.198 \pm 0.020	2.83 \pm 0.120	N.D.	N.D.
$\alpha_2\text{C}\text{AR}/\beta_2\text{AR}$	179 \pm 22	1.83 \pm 0.18	47.2 \pm 4.0	0.97 \pm 0.16	0.170 \pm 0.008	2.60 \pm 0.250	1.52 \pm 0.08	0.473 \pm 0.035
$\beta_2\text{AR}$	N.D.	N.D.	42.6 \pm 4.3	0.97 \pm 0.16	N.D.	N.D.	1.28 \pm 0.03	0.239 \pm 0.005

UK, UK 14,304; NE, norepinephrine; Iso, isoproterenol; Epi, epinephrine; N.D., no determination made.

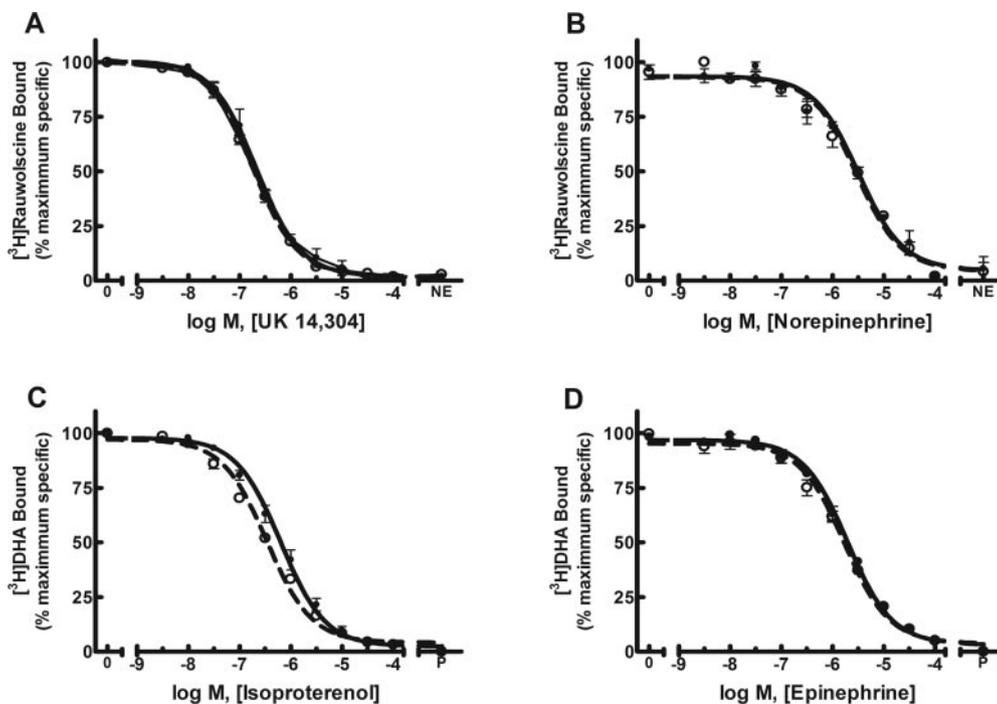


Fig. 5. Binding to agonists is not affected by coexpression of $\alpha_2\text{C}\text{AR}$ with $\beta_2\text{AR}$. Membranes from cells transiently expressing $\alpha_2\text{C}\text{AR}$ alone, $\alpha_2\text{C}\text{AR}/\beta_2\text{AR}$, or $\beta_2\text{AR}$ alone were prepared, and the ability of varying concentrations of the agonists UK 14,304 (A) and norepinephrine (B) to displace [^3H]rauwolscine binding sites or the agonists isoproterenol (C) and epinephrine (D) to compete for [^3H]DHA binding sites was investigated. Data shown are the average of three separate experiments.

suggesting cross-internalization between the two receptors (Fig. 7A).

The effect of coexpression with $\alpha_2\text{C}\text{AR}$ on $\beta_2\text{AR}$ internalization was also examined. As expected, a 30-min treatment with isoproterenol caused a robust 35% $\beta_2\text{AR}$ endocytosis, and this isoproterenol-induced internalization was not altered by coexpression of $\alpha_2\text{C}\text{AR}$. Unlike the apparent cross-internalization of $\alpha_2\text{C}\text{AR}$ following isoproterenol stimulation of coexpressed $\beta_2\text{AR}$, UK 14,304 stimulation of $\alpha_2\text{C}\text{AR}$ was unable to promote internalization of coexpressed $\beta_2\text{AR}$ (Fig. 7B).

Agonist-induced receptor internalization was also studied via confocal microscopy. When $\alpha_2\text{C}\text{AR}$ and $\beta_2\text{AR}$ were coexpressed and stimulated with isoproterenol, a loss of both receptors from the plasma membrane was observed, along with a concurrent accumulation of both receptors inside the cell (Fig. 8). In contrast, stimulation of the doubly transfected cells with UK 14,304 resulted in endocytosis of $\alpha_2\text{C}\text{AR}$ but not $\beta_2\text{AR}$ (data not shown). Thus, the data from the confocal studies matched the results from the luminometer-based assay described above well, in that both techniques revealed cointernalization of the $\alpha_2\text{C}\text{AR}/\beta_2\text{AR}$ complex upon treatment with $\beta_2\text{AR}$ agonists.

Discussion

A number of GPCRs, including $\text{GABA}_\text{B}\text{R1}$, $\alpha_{1\text{D}}\text{AR}$, $\alpha_2\text{C}\text{AR}$, and the olfactory receptors, are known to be inefficiently targeted to the cell surface when expressed heterologously in most cell types. Seminal studies demonstrating that coexpression with $\text{GABA}_\text{B}\text{R2}$ can facilitate $\text{GABA}_\text{B}\text{R1}$ trafficking to the cell surface suggested a key role for receptor heterodimerization in regulating the trafficking of certain GPCRs (Marshall et al., 1999). Likewise, associations of $\alpha_{1\text{D}}\text{AR}$ and olfactory receptors with specific GPCR partners have been found to enhance the surface expression of these trafficking-defective receptors (Uberty et al., 2003, 2005; Hague et al., 2004a,b). The purpose of the studies reported here was to investigate whether the poor trafficking of $\alpha_2\text{C}\text{AR}$ might also be enhanced by coexpression with an appropriate GPCR partner.

After examining $\alpha_2\text{C}\text{AR}$ surface trafficking following coexpression with more than 25 different GPCRs, we observed that surface expression of $\alpha_2\text{C}\text{AR}$ was markedly enhanced only by coexpression with $\beta_2\text{AR}$. Confocal microscopy studies confirmed increased surface expression of $\alpha_2\text{C}\text{AR}$ upon $\beta_2\text{AR}$ coexpression. We also observed that $\beta_2\text{AR}$ could be robustly

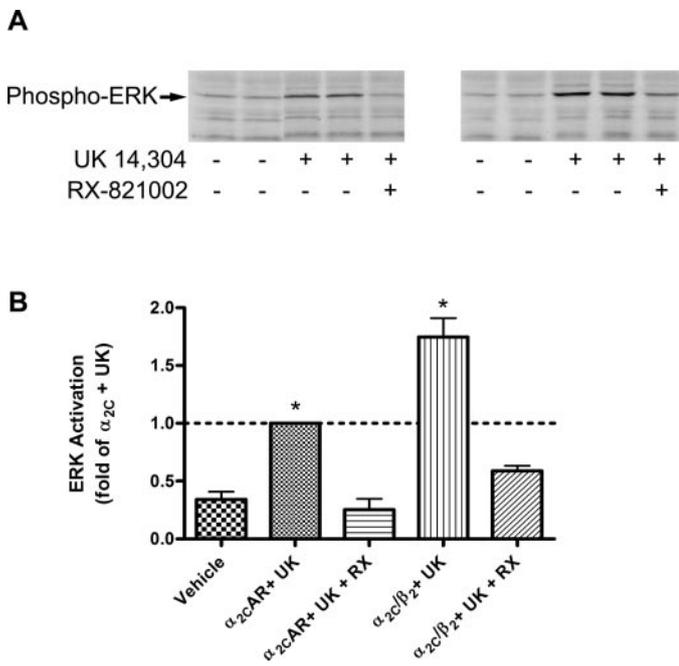


Fig. 6. Enhanced α_2C AR signaling upon coexpression with β_2 AR. **A**, HEK-293 cells transfected with α_2C AR in the absence or presence of β_2 AR were incubated with vehicle, UK 14,304 (10 μ M), or UK 14,304 with RX-821002 (10 μ M) for 5 min. Cells were harvested in 1 \times sample buffer, resolved by SDS-PAGE, and blotted for phospho-ERK1/2. **B**, the phosphorylated ERK1/2 bands from four separate experiments were quantified and normalized to total ERK1/2. *, $p < 0.05$.

coimmunoprecipitated with α_2C AR. Thus, a reasonable interpretation of these data is that α_2C AR surface expression is enhanced via association with β_2 AR, although it is not entirely clear whether the α_2C AR/ β_2 AR interaction is direct (via heterodimerization) or indirect (via joint interaction with a scaffold protein). In any case, the effects of β_2 AR coexpression on α_2C AR surface trafficking are analogous to previous observations that interactions with either α_{1B} AR or β_2 AR enable α_{1D} AR to localize normally to the plasma membrane (Uberti et al., 2003, 2005; Hague et al., 2004b). The effects of receptor coexpression on the trafficking of both α_2C AR and α_{1D} AR seem to be quite specific, because the vast majority of receptors examined had no significant effect on α_2C AR or α_{1D} AR surface expression. The interaction between GABA_BR1 and GABA_BR2 is also highly specific, as screens with several dozen other GPCRs revealed that only GABA_BR2 is capable of efficiently promoting GABA_BR1 surface trafficking (Balasubramanian et al., 2004).

Certain GPCR heterodimers exhibit altered pharmacology relative to the individual receptors expressed alone. For example, heterodimers formed between opioid receptors (κ/δ or μ/δ) possess ligand binding properties distinct from any of the three cloned opioid receptors expressed by themselves (Jordan and Devi, 1999; George et al., 2000). In our studies, coexpressed α_2C AR and β_2 AR did not seem to display altered affinities for any of the agonists or antagonists examined, suggesting that the conformation of the binding pockets for both receptors remained unaltered, as has been observed for other GPCR heterodimer combinations (Pfeiffer et al., 2002; Uberti et al., 2003). An increased B_{max} for [³H]rauwolscine binding was observed in saturation binding assays, where α_2C AR levels were increased by almost 2-fold when coexpressed with β_2 AR, and a similar increase was also observed

for α_2C AR immunoreactivity upon β_2 AR coexpression (data not shown). Increased receptor stability has been described for other trafficking-defective receptors upon coexpression with appropriate partners, such as α_{1D} AR coexpressed with α_{1B} AR (Uberti et al., 2003). The observed increases in α_2C AR levels upon β_2 AR coexpression might be explained by reduced α_2C AR retention in the endoplasmic reticulum, where accumulating α_2C AR would be rapidly degraded. Thus, because association with β_2 AR enhances the proportion of α_2C AR in the plasma membrane, it would reduce the amount of α_2C AR subject to rapid degradation and result in a modest but consistent increase in α_2C AR binding and immunoreactivity.

Receptor-receptor interactions are known to have strong effects on regulating signaling for certain GPCR combinations. In the case of trafficking-defective GPCRs, such as α_2C AR, associations with other receptors and the resultant-enhanced surface expression would seem to be critical due to the requirement for membrane-impermeant agonists to gain access to the receptors. In the current studies, UK 14,304-stimulated ERK1/2 activation by α_2C AR was found to be significantly increased upon coexpression with β_2 AR. The α_2 -specific nature of the ERK activation was shown by blocking α_2C AR with the specific antagonist RX 821002. Furthermore, α_2C AR stimulation of ERK phosphorylation, both in the absence and presence of β_2 AR coexpression, was fully blocked by pertussis toxin treatment (data not shown), suggesting predominant coupling of α_2C AR to G_{i/o}, even after association with β_2 AR. Thus, because α_2C AR ligand binding and G protein coupling specificity did not seem to be altered by coexpression with β_2 AR, the most plausible explanation for the enhanced signaling is that β_2 AR-induced trafficking of α_2C AR allowed for additional functional α_2C AR to be inserted into the plasma membrane.

The trafficking and functionality of α_2C AR are known to be heavily dependent on cellular context as well as the temperature at which cells are grown. Whereas α_2C AR is largely intracellular and nonfunctional in most heterologous cell types, it has been shown that α_2C AR is much more efficiently trafficked to the plasma membrane when expressed in certain neuronally derived cell lines (Hurt et al., 2000). It is tempting to speculate that the relative expression level of endogenous β_2 AR in these cell lines may be a key factor determining the trafficking and functionality of transfected α_2C AR, although of course, the relative expression levels of other proteins involved in regulating α_2C AR trafficking may also be very important. In various cell lines where transfected α_2C AR is poorly trafficked to the cell surface, it has been shown that lowering the temperature of the cells can promote α_2C AR plasma membrane expression (Jeyaraj et al., 2001; Bailey et al., 2004). Because the retention of misfolded proteins by the endoplasmic reticulum/Golgi complex is known to be less efficient at lower temperatures (Morello et al., 2000), it seems likely that an impairment in the ability of cells to retain α_2C AR accounts for the reported effect of temperature on α_2C AR trafficking. Whereas such temperature-dependent regulation of α_2C AR trafficking may occur in certain blood vessels in the distal limbs, temperatures low enough to help α_2C AR overcome its trafficking defect are unlikely to be achieved in most native cell types in which α_2C AR is expressed. Thus, it seems probable that α_2C AR trafficking and functionality in vivo are dependent on cellu-

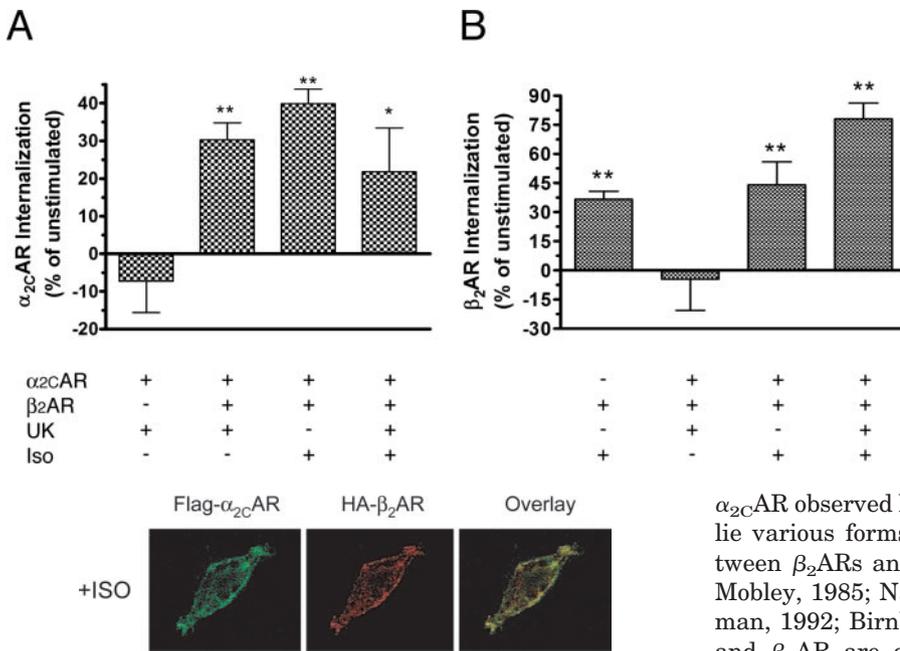


Fig. 8. Confocal microscopy analysis of α_{2C} AR cointernalization with β_2 AR. In HEK-293 cells, α_{2C} AR and β_2 AR were coexpressed and stimulated with isoproterenol (10 μ M) for 30 min. FLAG- α_{2C} AR (green) and HA- β_2 AR (red) were visualized using secondary antibodies coupled to Alexa 488 or Alexa 546. For comparison with unstimulated cells, compare these data with Fig. 2, C to E. The data shown in this figure are representative of three separate experiments.

lar factors, such as associations with other receptors as reported here and/or interactions with accessory proteins that promote proper receptor trafficking.

The regulation of α_{2C} AR by agonist-promoted internalization has been difficult to study because of the poor surface expression of the receptor, although some progress has been made using ELISA-based assays similar to those used in the present studies (Daunt et al., 1997; DeGraff et al., 1999; Olli-Lahdesmaki et al., 1999). Results from previous studies suggested that, in Madin-Darby canine kidney cells, α_{2C} AR was weakly internalized in response to agonist (Daunt et al., 1997), whereas in COS-1 cells, α_{2C} AR internalization was not observed unless arrestin-3 was overexpressed (DeGraff et al., 1999). Because β_2 AR cotransfection robustly increased α_{2C} AR surface expression in our studies, we took advantage of the opportunity to characterize the internalization properties of α_{2C} AR in response to agonist. Furthermore, because α_{2C} AR and β_2 AR associate in cells, we also assessed the consequences of this interaction for receptor endocytosis. We found that α_{2C} AR was significantly internalized in response to UK 14,304, only when α_{2C} AR was coexpressed with β_2 AR. We also observed a marked internalization of α_{2C} AR in response to isoproterenol, indicating that α_{2C} AR undergoes cointernalization with β_2 AR upon β_2 AR agonist stimulation. These findings were confirmed by confocal microscopy studies, which showed colocalization of α_{2C} AR and β_2 AR in intracellular punctate regions following stimulation with isoproterenol. Interestingly, as with the luminometer assays, internalization of β_2 AR did not seem to be affected by UK 14,304 treatment, which may indicate that recruitment of arrestin to the α_{2C} AR/ β_2 AR complex is dependent on whether the α_{2C} AR component or β_2 AR component is stimulated by agonist. The isoproterenol-stimulated internalization of

Fig. 7. Cointernalization of α_{2C} AR and β_2 AR. A, cells transfected with FLAG- α_{2C} AR were incubated with UK 14,304 (10 μ M) or isoproterenol (ISO, 10 μ M) for 30 min in the presence or absence of coexpression with HA- β_2 AR. The dishes were placed on ice, washed twice, and fixed. Internalization was defined as the loss of FLAG- α_{2C} AR from the cell surface using a luminometer-based assay. B, cells transfected with HA- β_2 AR were incubated with UK 14,304 or ISO for 30 min in the presence or absence of coexpression with FLAG- α_{2C} AR. The dishes were placed on ice, washed twice, and fixed. Internalization was defined as the loss of HA- β_2 AR from the cell surface using the luminometer-based assay. Data shown are from four separate experiments. Asterisks indicate significant differences from unstimulated cells. *, $p < 0.05$; **, $p < 0.01$.

α_{2C} AR observed here suggests a mechanism that may underlie various forms of cross-talk that have been reported between β_2 ARs and α_2 ARs (Maggi et al., 1980; Northam and Mobley, 1985; Nakamura et al., 1991; Atkinson and Minneman, 1992; Birnbaum et al., 1995). It is known that α_{2C} AR and β_2 AR are coexpressed in many of the same tissues, including distinct structures within the brain, adrenal glands, and kidney (Rainbow et al., 1984; Rosin et al., 1996; Lee et al., 1998; Uhlen et al., 1998; Brede et al., 2003; Cesetti et al., 2003; Wallace et al., 2004). Further investigations into the consequences of α_{2C} AR/ β_2 AR associations in native tissues, e.g., studies on knockout mice, may shed additional light on the physiological importance of the interaction between these receptors in vivo.

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