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Heterodimerization with β_2-Adrenergic Receptors Promotes Surface Expression and Functional Activity of α_{1D}-Adrenergic Receptors

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ABSTRACT

The α_{1D}-adrenergic receptor (α_{1D}-AR) is a G protein-coupled receptor (GPCR) that is poorly trafficked to the cell surface and largely nonfunctional when heterologously expressed by itself in a variety of cell types. We screened a library of approximately 30 other group I GPCRs in a quantitative luminometer assay for the ability to promote α_{1D}-AR cell surface expression. Strikingly, these screens revealed only two receptors capable of inducing robust increases in the amount of α_{1D}-AR at the cell surface: α_{1B}-AR and β_2-AR. Confocal imaging confirmed that coexpression with β_2-AR resulted in translocation of α_{1D}-AR from intracellular sites to the plasma membrane. Additionally, coimmunoprecipitation studies demonstrated that α_{1D}-AR and β_2-AR specifically interact to form heterodimers when coexpressed in HEK-293 cells. Ligand binding studies revealed an increase in total α_{1D}-AR binding sites upon coexpression with β_2-AR, but no apparent effect on the pharmacological properties of the receptors. In functional studies, coexpression with β_2-AR significantly enhanced the coupling of α_{1D}-AR to norepinephrine-stimulated Ca^{2+} mobilization. Heterodimerization of β_2-AR with α_{1D}-AR also conferred the ability of α_{1D}-AR to cointernalize upon β_2-AR agonist stimulation, revealing a novel mechanism by which these different adrenergic receptor subtypes may regulate each other's activity. These findings demonstrate that the selective association of α_{1D}-AR with other receptors is crucial for receptor surface expression and function and also shed light on a novel mechanism of cross talk between α_1- and β_2-ARs that is mediated through heterodimerization and cross-internalization.

Adrenergic receptors (ARs) mediate physiological responses to the catecholamines norepinephrine (NE) and epinephrine. ARs are subdivided into three major families (α_1, α_2, and β) based on their structure, pharmacology, and signaling mechanisms (Hieble et al., 1995). At least three closely related subtypes have been identified within each family, with each subtype differentially expressed in various tissues. ARs are members of the rhodopsin-like group I G protein-coupled receptor (GPCR) superfamily and exhibit the characteristic GPCR architecture featuring seven membrane-spanning domains.

Traditionally, GPCRs have been thought to function as monomers, but a significant amount of recent evidence suggests that GPCRs can also exist as dimers consisting of identical or distinct monomeric subunits. Dimerization of GPCRs may alter the receptors' functional, pharmacological, or regulatory properties and, in some cases, may be absolutely required for receptor function (Angers et al., 2002). For example, two nonfunctional $GABA_B$ receptors seem to form an obligate heterodimer that is necessary for cell surface expression and functional $GABA_B$ receptor activity (Marshall et al., 1999). Sweet and umami taste receptors also seem to form obligate heterodimers (Zhao et al., 2003). Many GPCRs are known to exhibit poor surface expression and functionality when expressed alone in heterologous cells (Tan et al., 2004), but beyond the examples of $GABA_B$

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ABBREVIATIONS: AR, adrenergic receptor; NE, norepinephrine; GPCR, G protein-coupled receptor; HA, hemagglutinin; GFP, green fluorescent protein; HEK, human embryonic kidney; DβM, *n*-dodecyl-β-D-maltoside; BMY 7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; HRP, horseradish peroxidase; ECL, enzyme-linked chemiluminescence; PBS, phosphate-buffered saline; BE, 2-[β-(4-hydroxyphenyl)-ethylaminomethyl]-tetralone; CGP-12177, 4-[3-[(1,1-dimethyethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one; CGP-20712A, (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(methylethyl)amino-2-butanol.

and taste receptors, the generality of GPCR heterodimerization in controlling receptor surface expression has not been widely explored.

The α_{1D}-AR is a well documented example of a GPCR that is poorly expressed at the cell surface and largely nonfunctional when heterologously expressed alone in most cell types (Theroux et al., 1996; Chalothorn et al., 2002). Interestingly, we have recently found that heterodimerization with the closely related α_{1B}-AR results in robust surface expression of the normally intracellular α_{1D}-AR while also increasing α_{1D}-AR responsiveness to NE and promoting agonist-induced α_{1D}-AR internalization (Uberti et al., 2003; Hague et al., 2004b). These studies suggested the possibility that heterodimerization with α_{1B}-AR might be required for α_{1D}-AR function. However, other recent studies utilizing double α_{1A}-/α_{1B}-AR knockout mice have revealed the presence of functional α_{1D}-AR, even in the absence of other α_1-AR subtypes (Turnbull et al., 2003). Additionally, α_{1D}-AR is found in some tissues that lack α_{1B}-AR expression such as human bladder and specific regions of the spinal cord and brain, as well as certain rat and human blood vessels (Alonso-Llamazares et al., 1995; Michelotti et al., 2000; Tanoue et al., 2002; Sadalge et al., 2003). Thus, it is reasonable to speculate that trafficking of functional α_{1D}-AR to the cell surface may involve the interaction of α_{1D}-AR with other proteins beyond α_{1B}-AR. In the present study, we screened a library of approximately 30 group I GPCRs for possible interacting partners that might be able to traffic α_{1D}-AR to the cell surface in a functional manner.

Methods and Materials

Plasmids and Other Materials. Epitope-tagged (Flag- and HA-tagged) versions of human α_{1A}-, α_{1B}-, and α_{1D}-AR cDNAs have been described previously (Vicentic et al., 2002; Uberti et al., 2003). Human α_{1D}-AR C-terminally tagged GFP construct in pEGFP-N3 was kindly provided by Gozoh Tsujimoto (National Children's Hospital, Tokyo, Japan). HA-tagged β_1- and β_2-AR cDNAs were kindly provided by Hitoshi Kurose (Kyushu University, Japan). HA-tagged α_{2A}-, α_{2B}-, and α_{2C}-AR cDNAs were kindly provided by Lee Limbird (Vanderbilt University Medical Center, Nashville, TN). Flag-tagged Dopamine1 and Dopamine2 receptor cDNAs were kindly provided by David Sibley (National Institutes of Health, Bethesda, MD). HA-tagged serotonin 5HT1A receptor cDNA was kindly provided by John Raymond (Medical University of South Carolina, Charleston, SC). Flag-tagged angiotensin AT1 and AT2 receptor cDNAs were kindly provided by Victor Dzau (Harvard Medical School, Boston, MA). HA-tagged muscarinic m1–5 acetylcholine receptor cDNAs were kindly provided by Allen Levey (Emory University School of Medicine, Atlanta, GA). HA-tagged opioid receptor cDNAs (μ and δ) were kindly provided by Ping-Yee Law (University of Minnesota School of Medicine, Duluth, MN). Flag-tagged lysophosphatidic acid lysophosphatidic acid-1 and -2 receptor cDNAs were kindly provided by Jerold Chun (University of California, San Diego, CA). Flag-tagged histamine H1–3 receptor cDNAs were kindly provided by Rob Leurs (Vrije Universiteit, The Netherlands). HA-tagged melatonin MT1 and Myc-tagged melatonin MT2 receptor cDNAs were kindly provided by Tarfa Kokkola (University of Kuopio, Finland). Flag-tagged melatonin-related receptor cDNA was kindly provided by Peter J. Morgan (Rowett Research Institute, Scotland), and HA-tagged purinergic P2Y1 receptor cDNA was kindly provided by Michael Salter (University of Toronto, Canada). For the screens examining the effects of receptor coexpression on α_{1D}-AR surface expression, HA-α_{1D}-AR was utilized in cases where the coexpressed receptor was Flag-tagged, whereas Flag-α_{1D}-AR was utilized in cases where the coexpressed receptor was either HA- or Myc-tagged. Expression of the receptors was verified via Western blotting.

Other materials were obtained from the following sources: HEK-293 cells (American Type Culture Collection, Manassas, VA); fura-2/acetoxymethly ester and *n*-dodecyl-β-D-maltoside (DβM) (Calbiochem, San Diego, CA); (−)-norepinephrine bitartrate, BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride), prazosin, albuterol, Dulbecco's modified Eagle's medium (DMEM), penicillin, concanavalin A, streptomycin, bovine serum albumin (BSA), anti-FLAG M2 affinity resin, and HRP-conjugated anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO); anti-HA affinity matrix and 12CA5 anti-HA monoclonal antibody (Roche Diagnostics, Indianapolis, IN); and ECL reagent and enzyme-linked immunosorbent assay SuperSignal Pico ECL (Pierce Chemical, Rockford, IL). LipofectAMINE 2000 reagent and all electrophoresis reagents and precast 4 to 20% Tris-Glycine polyacrylamide gels were obtained from Invitrogen (Carlsbad, CA).

Cell Culture and Transfections. HEK-293 cells were maintained in DMEM, supplemented with 10% fetal bovine serum, 10 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% CO_2. For heterologous expression of receptors, 2 to 4 μg of cDNA was mixed with LipofectAMINE 2000 (15 μl) and added to 5 ml of serum-free medium in 10-cm plates containing cells at 70 to 90% confluency for 16 h, followed by a change of medium. Cells were harvested 48 to 72 h after transfection.

Surface Expression Assay. HEK-293 cells were transiently transfected with the appropriate epitope-tagged constructs with LipofectAMINE 2000 as described above. After 24 h, cells were split into poly-D-lysine-coated 35-mm dishes and grown overnight at 37°C to 80 to 90% confluency. Cells were then rinsed three times with phosphate-buffered saline (PBS) with Ca^{2+}, fixed with 4% paraformaldehyde in PBS/Ca^{2+} for 30 min, and rinsed three times with PBS/Ca^{2+}. Cells were then incubated in blocking buffer (2% nonfat milk in PBS/Ca^{2+}) for 30 min and incubated with the appropriate concentrations of HRP-conjugated anti-FLAG M2 or 12CA5 anti-HA monoclonal antibodies in blocking buffer for 1 h at room temperature. Following incubation with the HRP-conjugated anti-Flag antibody, cells were washed three times with blocking buffer, one time with PBS/Ca^{2+}, and then incubated with enzyme-linked immunosorbent assay ECL reagent for 15 sec. Following incubation with the 12CA5 antibody, cells were washed three times with blocking buffer and incubated with the appropriate concentration of HRP-conjugated anti-mouse secondary antibody for 1 h, washed, and then incubated with ECL. The chemiluminescence of the whole 35-mm plate, which corresponds to the amount of receptor on the cell surface (Uberti et al., 2003), was quantified in a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). Control experiments using anti-tubulin antibodies revealed no detectable luminescence, revealing no antibody penetration of the cells under the fixation conditions that were used. For each data point, three to five plates were averaged per experiment. The results were analyzed using unpaired Student's *t* tests where applicable (GraphPad Software Inc., San Diego, CA). For internalization assays, cells were first rinsed and then stimulated with or without 10 μM albuterol in DMEM for 30 min at 37°C before cell surface measurements described above. Mean values ± S.E.M. were calculated as percent absorbance in arbitrary units and statistically compared using the unpaired Student's *t* test, with a p value less than 0.05 considered significant.

Confocal Microscopy. Cells transiently transfected with HA- or GFP-tagged constructs were grown on sterile coverslips, fixed with 4% paraformaldehyde, and permeabilized with saponin buffer containing 2% BSA and 0.04% saponin in PBS for 30 min at room temperature. The cells were then incubated with 12CA5 anti-HA monoclonal antibody for 1 h at room temperature. After three washes with saponin buffer, cells were incubated with a rhodamine red-conjugated anti-mouse IgG at a 1:200 dilution for 1 h at room temperature. After three washes with saponin buffer and one wash with PBS, coverslips were mounted using Vectashield mounting medium

(Vector Laboratories (Burlingame, CA). Cells were scanned with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss GmbH, Jena, Germany) as described previously (Uberti et al., 2003; Hague et al., 2004b). For detection of GFP, fluorescein isothiocyanate fluorescence was excited using an argon laser at a wavelength of 488 nm. The absorbed wavelength was detected for 510 to 520 nm for GFP. For detecting rhodamine red, rhodamine fluorescence was excited using a helium-neon laser at a wavelength of 522 nm. The pinhole size was maintained at 1 airy unit for all images.

Radioligand Binding. For radioligand binding, confluent 15-cm plates were washed with PBS and harvested by scraping. Cells were collected by centrifugation and homogenized with a Polytron. Cell membranes were collected by centrifugation at 30,000*g* for 20 min and resuspended by homogenization in 1× buffer (25 mM HEPES, 150 mM NaCl, pH 7.4, and 5 mM EDTA) with a protease inhibitor cocktail (1 mM benzamidine, 3 μM pepstatin, 3 μM phenylmethylsulfonylfluoride, 3 μM aprotinin, and 3 μM leupeptin). Radioligand binding sites were measured by saturation analysis of the specific binding of the α_1-AR antagonist radioligand ^{125}I-BE (20–800 pM) or the β-AR antagonist ^{125}I-pindolol (40–2000 pM). Nonspecific binding was defined as binding in the presence of 10 μM phentolamine (for ^{125}I-BE) or 100 μM isoproterenol (for ^{125}I-pindolol). The pharmacological specificity of α_{1D}-AR binding sites was determined by displacement of ^{125}I-BE (50 pM) by NE, a nonselective adrenergic agonist, prazosin, a nonselective α_1-AR antagonist, or BMY 7378, a selective α_{1D}-AR antagonist. The pharmacological specificity of β_2-AR binding sites was determined by displacement of ^{125}I-pindolol (100 pM) by the β-AR-selective ligands CGP-12177 (4-[3-[(1,1-dimethyethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one), CGP-20712A [(±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt], and ICI 118,551 [(±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(methylethyl)amino-2-butanol]. Data were analyzed by nonlinear regression analysis (Theroux et al., 1996).

Solubilization, Immunoprecipitation, and Western Blot Analysis. Membrane preparations (2–3 mg of protein) were prepared as described above and solubilized with 2% DβM in 1× buffer supplemented with a protease inhibitor cocktail for 2 h at 4°C with gentle agitation. Following solubilization, samples were centrifuged at 16,000*g*, and supernatants were diluted to 0.2% DβM in 1× buffer supplemented with protease inhibitors. Soluble receptors were incubated with M2 anti-FLAG or anti-HA affinity matrix overnight at 4°C with gentle agitation. Resin was collected by centrifugation, washed with 1× buffer, and then eluted with 4× sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 5% β-mercaptoethanol).

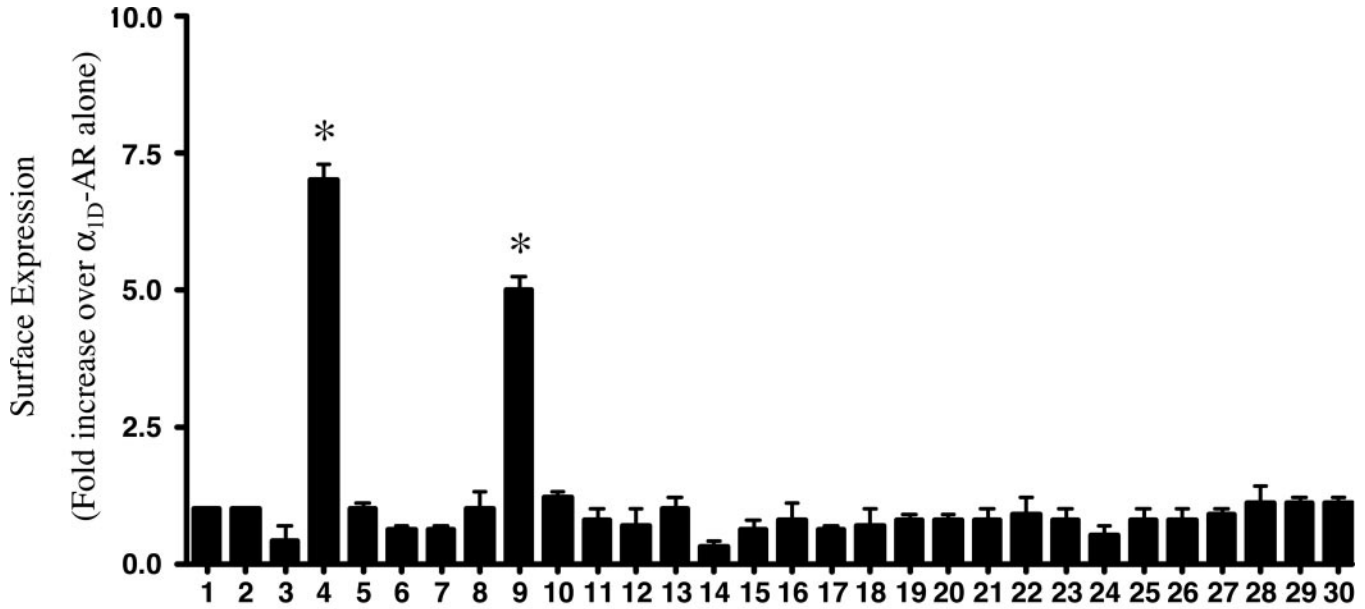
Immunoprecipitated samples were run on a 4 to 20% Tris-Glycine SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature with gentle agitation. Membranes were then incubated with the appropriate concentration of HRP-conjugated M2-anti-FLAG antibody or 12CA5 anti-HA monoclonal antibody for 1 h at room temperature. Membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and detected with ECL directly or alternatively incubated with the appropriate concentration of secondary IgG antibody and then detected with ECL.

Measurement of Intracellular Calcium Mobilization. Intracellular Ca^{2+} mobilization was measured using fura-2 as described previously (Theroux et al., 1996). In brief, confluent 15-cm plates of transiently transfected HEK-293 cells were washed one time with Ca^{2+}-free Hanks solution and then detached using 0.25% trypsin. Cells were collected in 10 ml of Hanks buffer with Ca^{2+} and centrifuged for 2 min at 1000*g* at 4°C. Cells were resuspended in DMEM containing 0.05% BSA and incubated with 1 μM fura-2/AM for 15 min. Cells were then diluted, centrifuged and resuspended in biological salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, and 0.1% BSA), and divided into 3-ml aliquots (2.0×10^6 cells/ml) and placed on ice. Prior to use, cells were warmed to 37°C, centrifuged, resuspended in 3 ml of biological salt solution, and transferred to a cuvette. Luminescence was measured by a PerkinElmer LS50 luminescence spectrofluorometer (PerkinElmer Life and Analytical Sciences (Boston, MA). NE (100 μM) was used to stimulate α_{1D}-AR-induced Ca^{2+} mobilization. Calculation of $[Ca^{2+}]_i$ was performed by equilibrating intra- and extracellular Ca^{2+} with 30 μM digitonin (R_{max}) followed by 9 mM EGTA (R_{min}) using a K_d of 225 nM for fura-2. Mean values ± S.E.M. were calculated and were statistically compared using the unpaired Student's *t* test, with a *p* value less than 0.05 considered significant. For desensitization assays, cells were pretreated with or without 10 μM albuterol in DMEM for 30 min at 37°C. Cells were then rinsed three times with Ca^{2+}-free Hanks buffer, and Ca^{2+} mobilization was measured as described above.

Results

β_2-AR Promotes α_{1D}-AR Surface Expression. Previous studies have revealed that α_{1D}-AR is primarily found in intracellular compartments when expressed in a variety of heterologous cells (Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002; Hague et al., 2004b). Recently, we found that α_{1B}-/α_{1D}-AR heterodimerization can dramatically increase the surface expression and functional activity of α_{1D}-AR (Uberti et al., 2003; Hague et al., 2004b). To screen for other potential interacting receptors that might traffic α_{1D}-AR to the plasma membrane, we used a luminometer-based surface expression assay, which we have previously used to examine the surface expression of epitope-tagged GPCRs (Uberti et al., 2003; Hague et al., 2004b). In this assay, Flag- or HA-tagged α_{1D}-AR was coexpressed with various group I GPCRs in HEK-293 cells, and the cell surface expression of the α_{1D}-AR was quantified (Fig. 1). When expressed alone, α_{1D}-AR was barely detectable on the cell surface, whereas coexpression with α_{1B}-AR significantly increased α_{1D}-AR surface expression, as previously reported (Uberti et al., 2003; Hague et al., 2004b). α_{1D}-AR was also coexpressed in these screens with 28 other GPCRs. Strikingly, the only receptor other than α_{1B}-AR to have an effect on α_{1D}-AR surface expression was β_2-AR, which produced nearly as dramatic an enhancement of α_{1D}-AR trafficking as α_{1B}-AR. In contrast, other members of the AR family and all of the other group 1 GPCRs examined did not have any detectable effect on trafficking of α_{1D}-AR to the cell surface. These results reveal a novel and selective effect of β_2-AR on facilitating efficient plasma membrane trafficking of α_{1D}-AR, similar to the effect observed previously with α_{1B}-AR.

To confirm these results using a different technique, we performed immunofluorescence confocal microscopy experiments. HEK-293 cells were cotransfected with GFP-tagged α_{1D}-AR and HA-tagged β_2-AR (Fig. 2, D–F) or HA-tagged β_1-AR (Fig. 2, G–I). α_{1D}-AR was found to be localized intracellularly when expressed alone (Fig. 2, A–C). However, as seen in Fig. 2, D to F, coexpression with β_2-AR resulted in a dramatic translocation of α_{1D}-AR from intracellular sites to the plasma membrane. In contrast, coexpression of β_1-AR with α_{1D}-AR did not result in any significant translocation of α_{1D}-AR, which remained predominantly intracellular, whereas β_1-AR immunostaining was found almost exclusively on the cell surface (Fig. 2, G–I). These data confirm that β_2-AR, but not β_1-AR, selectively promotes trafficking of α_{1D}-AR to the plasma membrane.



1. α_{1D}AR-FLAG	6. α_{1D} + α_{2B}AR	11. α_{1D} + AT_2	16. α_{1D} + LPA_2	21. α_{1D} + MT1	26. α_{1D} + M1
2. α_{1D}AR-HA	7. α_{1D} + α_{2C}AR	12. α_{1D} + H1	17. α_{1D} + μOR	22. α_{1D} + MT2	27. α_{1D} + M2
3. α_{1D} + α_{1A}AR	8. α_{1D} + β_1AR	13. α_{1D} + H2	18. α_{1D} + δOR	23. α_{1D} + MRR	28. α_{1D} + M3
4. α_{1D} + α_{1B}AR	9. α_{1D} + β_2AR	14. α_{1D} + H3	19. α_{1D} + D1	24. α_{1D} + P_2Y_1	29. α_{1D} + M4
5. α_{1D} + α_{2A}AR	10. α_{1D} + AT_1	15. α_{1D} + LPA_1	20. α_{1D} + D2	25. α_{1D} + $5HT_1$	30. α_{1D} + M5

Fig. 1. α_{1D}-AR cell surface expression is promoted by coexpression with α_{1B}-AR and β_2-AR but not by coexpression with other group I GPCRs. Flag- or HA-tagged α_{1D}-AR surface expression was detected and quantified by a luminometer-based expression assay following coexpression in HEK-293 cells with a variety of other group I GPCRs, as listed (1–30). The receptors examined in this study were chosen because they are all group I GPCRs that are known to be found in at least some of the same tissues as α_{1D}-AR. In these experiments, α_{1D}-AR expressed alone was barely detectable on the cell surface, whereas α_{1D}-AR coexpressed with α_{1B}-AR showed a 6- to 8-fold increase in α_{1D}-AR surface expression, as previously reported. Coexpression with most of the other group I GPCRs examined had no significant effect on α_{1D}-AR surface expression, but coexpression with β_2-AR resulted in a significant (5- to 6-fold) increase in α_{1D}-AR surface expression. The bars represent the mean fold increase in α_{1D}-AR surface expression for each coexpressed receptor relative to α_{1D}-AR alone, and each error bar represents the S.E.M. for three to six independent experiments. *, significantly different ($p < 0.05$) relative to α_{1D}-AR expressed alone, as assessed using an unpaired Student's t test analysis. AT, angiotensin receptor; LPA, lysophosphatidic acid receptor; D, dopamine receptor; H, histamine receptor; OR, opioid receptor; MT, melatonin receptor; MRR, melatonin-related receptor; M, muscarinic acetylcholine receptor; 5HT, serotonin receptor; P2Y, purinergic receptor.

β_2-AR Physically Associates with α_{1D}-AR in HEK-293 Cells. The strong enhancement of α_{1D}-AR surface expression induced by coexpression with β_2-AR suggested the possibility of a physical association between the two receptors. Coimmunoprecipitation experiments using differentially tagged receptors were performed to test this hypothesis. Flag-tagged α_{1D}-AR was expressed either alone or in combination with HA-tagged β_2- or β_1-AR in HEK-293 cells (Fig. 3). As expected, Western blotting for Flag-tagged α_{1D}-AR after immunoprecipitation with anti-Flag antibody revealed the presence of α_{1D}-AR (data not shown). Interestingly, HA-tagged β_2-AR was robustly coimmunoprecipitated with Flag-tagged α_{1D}-AR (Fig. 3B, lane 2). In contrast, HA-tagged β_1-AR did not detectably coimmunoprecipitate with α_{1D}-AR (Fig. 3B, lane 3). The lack of α_{1D}-/β_1-AR coimmunoprecipitation was not due to inefficient receptor expression because parallel blots of cell lysates showed a comparable level of expression for both HA-tagged β_1- and β_2-AR (Fig. 3A). Furthermore, α_{1D}-AR/β_2-AR interactions were not detected when the receptors were expressed in separate populations of cells that were solubilized, sonicated, and mixed together prior to immunoprecipitation and Western blot analysis (data not shown). Taken together, these data indicate that α_{1D}-AR and β_2-AR exhibit selective heterodimerization in a cellular context.

Pharmacological Properties of α_{1D}-/β_2-AR Heterodimers. To determine whether the physical interaction of α_{1D}-AR with β_2-AR might result in altered α_{1D}-AR pharmacological properties, ligand binding studies were performed using the α_1-AR-selective antagonist ^{125}I-BE. The density of binding sites (B_{max}) and the affinity (K_d) for ^{125}I-BE were determined via saturation analysis of specific ^{125}I-BE binding to membranes expressing either α_{1D}-AR alone or α_{1D}-/β_2-AR (Table 1). No significant difference in affinity for ^{125}I-BE binding was observed upon coexpression of α_{1D}-AR with β_2-AR. However, the density of α_{1D}-AR binding sites increased by nearly 2-fold upon coexpression of the two receptors (Table 1), similar to what has previously been observed for α_{1D}-AR coexpression with α_{1B}-AR (Uberti et al., 2003). These data are consistent with the observed effects of β_2-AR and α_{1B}-AR on promoting the surface expression of α_{1D}-AR, since it is known that properly assembled multi-

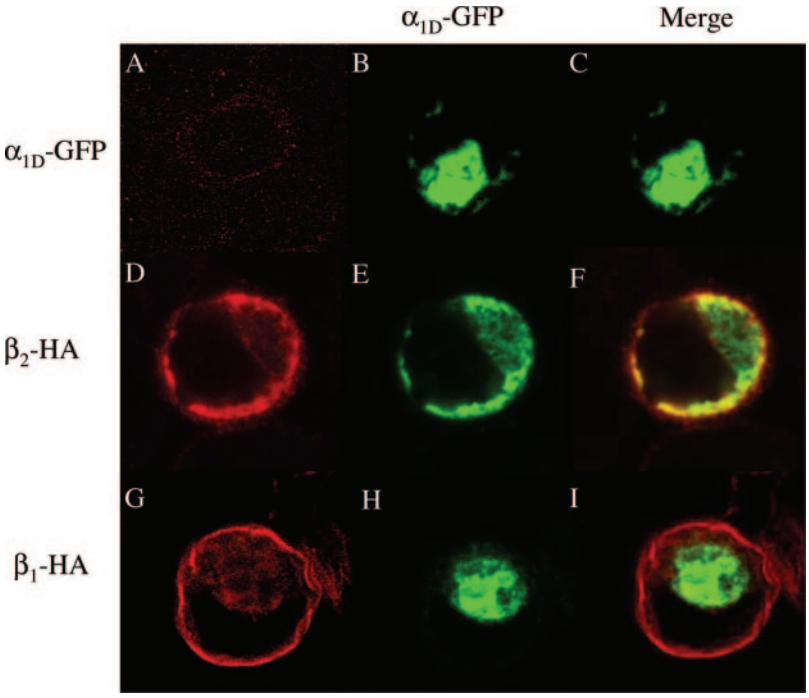


Fig. 2. Coexpression of α_{1D}-AR with β_2-AR results in plasma membrane expression and colocalization of α_{1D}-/β_2-AR. C-terminal GFP-tagged α_{1D}-AR expressed alone in HEK-293 cells was found predominantly in intracellular compartments, as shown via confocal microscopy (A–C). Coexpression with HA-β_2-AR, which was visualized with rhodamine red, resulted in markedly enhanced membrane targeting of α_{1D}-AR and colocalization of the receptors (D–F). When GFP-α_{1D}-AR was coexpressed with HA-β_1-AR, in contrast, α_{1D}-AR remained localized intracellularly, whereas β_1-AR was found almost exclusively at the plasma membrane (G–I). Each image is representative of a number of cells examined from three to four individual experiments for each condition.

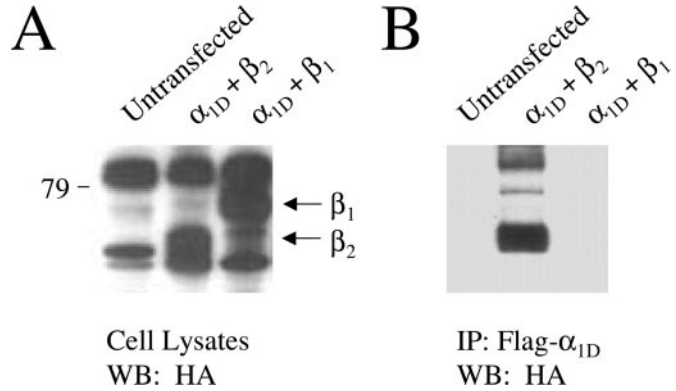


Fig. 3. Coimmunoprecipitation of β_2-AR but not β_1-AR with α_{1D}-AR. A, Western blots of HEK-293 cell lysates coexpressing Flag-α_{1D}-AR with HA-β_1- or HA-β_2-AR. Expression levels of the two β-AR subtypes were examined with an anti-HA antibody. The anti-HA antibody detects several nonspecific bands, as shown in the first lane (Untransfected), but the arrows indicate the bands corresponding to transfection-specific detection of β_1- and β_2-AR, which were expressed at comparable levels. B, Flag-α_{1D}-AR was expressed alone or coexpressed with either HA-β_1- or HA-β_2-AR in HEK-293 cells. Membranes were solubilized and immunoprecipitated (IP) with anti-Flag affinity resin, and Western blots were performed with the anti-HA antibody. β_2-AR was robustly coimmunoprecipitated with α_{1D}-AR, whereas β_1-AR was not. The data from all panels of this figure are representative of three to four experiments for each condition.

meric proteins residing in the plasma membrane often exhibit much slower rates of turnover than unassembled proteins that are trapped in the ER/Golgi complex (Wanamaker et al., 2003). Further pharmacological studies were performed to assess the inhibition of specific ^{125}I-BE binding by the endogenous ligand NE, the α_1-AR-selective antagonist prazosin, and the α_{1D}-selective antagonist BMY 7378. The K_i values for all three of these ligands were not significantly different for membranes expressing α_{1D}-AR alone versus membranes expressing the α_{1D}-AR/β_2-AR combination (Table 1). Similarly, no changes in binding affinity were observed for several β-AR-selective ligands in displacing ^{125}I-pindolol binding to membranes expressing β_2-AR alone versus membranes expressing both α_{1D}-AR and β_2-AR (Table 2). Thus, heterodimerization of β_2-AR with α_{1D}-AR was not found in these studies to alter the limited number of receptor pharmacological properties that were examined.

TABLE 1

Ligand binding properties of α_{1D}-AR alone versus α_{1D}-/β_2-AR

HEK-293 cells were either transfected with α_{1D}-AR alone or cotransfected with α_{1D}-AR and β_2-AR. Specific binding of [^{125}I]-BE was measured as described under *Materials and Methods*. B_{max} and K_d values were calculated by nonlinear regression of saturation curves, and K_i values for inhibition of binding by BMY7378, prazosin, and NE were also determined. Each value represents the mean ± S.E.M. for three to six experiments in duplicate.

	α_{1D}-AR	α_{1D}-/β_2-AR
[^{125}I]BE (K_d)	74 ± 11 pM	73 ± 8 pM
[^{125}I]BE (B_{max})	130 ± 13 fmol/mg	252 ± 17 fmol/mg*
BMY7378 (K_i)	18 ± 6 nM	14 ± 4 nM
Prazosin (K_i)	0.33 ± 0.04 nM	0.43 ± 0.09 nM
Norepinephrine (K_i)	820 ± 80 nM	870 ± 60 nM

* Significantly different ($P < 0.05$) compared with α_{1D}-AR expressed alone.

Increased Functional Responses and Internalization of α_{1D}-/β_2-AR Heterodimers. Since coexpression with β_2-AR enhanced α_{1D}-AR surface expression and binding site density, we next determined if this physical interaction might increase α_{1D}-AR functional responses. β_2-AR and α_{1D}-AR are known to be primarily coupled to different G

TABLE 2

Ligand-binding properties of β_2-AR alone versus α_{1D}-/β_2-AR

HEK-293 cells were either transfected with α_{1D}-AR alone or cotransfected with α_{1D}-AR and β_2-AR. Specific binding of [^{125}I]-pindolol was measured as described under *Materials and Methods*. B_{max} and K_d values were calculated by nonlinear regression of saturation curves, and K_i values for inhibition of binding by unlabeled CGP-12177A, CGP-20712, and ICI 118,551 were determined. Each value represents the mean ± S.E.M. for three to four experiments performed in duplicate.

	β_2-AR	α_{1D}-/β_2-AR
[^{125}I]Pindolol (K_d)	244 ± 51 pM	359 ± 87 pM
[^{125}I]Pindolol (B_{max})	328 ± 40 fmol/mg	358 ± 46 fmol/mg
(±)CGP-12177A (K_i)	5.1 ± 1.4 nM	8.7 ± 2.3 nM
CGP-20712 (K_i)	15.7 ± 2.1 nM	13.6 ± 3.6 nM
ICI 118,551 (K_i)	3.5 ± 0.9 nM	7.1 ± 2.9 nM

proteins (G_s and G_q, respectively). We examined α_{1D}-AR signaling by studying NE-induced intracellular Ca^{2+} mobilization. It has previously been reported that α_{1D}-AR transfection into certain cell types results in a modest amount of constitutive receptor activity (Garcia-Sainz and Torres-Padilla, 1999; McCune et al., 2000), but we did not observe any evidence for agonist-independent receptor signaling upon α_{1D}-AR transfection into HEK-293 cells. Furthermore, α_{1D}-AR surface expression was not enhanced by treatment of the cells with prazosin in our experiments (data not shown). When cells expressing α_{1D}-AR alone were stimulated with NE, a marginal amount of Ca^{2+} mobilization was observed, as previously reported (Hague et al., 2004b). However, there was a substantial (~2.5-fold) increase in NE-stimulated Ca^{2+} mobilization when β_2-AR was coexpressed α_{1D}-AR (Fig. 4A). No significant Ca^{2+} mobilization was observed in cells transfected with β_2-AR alone (data not shown). Since many GPCRs are known to undergo internalization from the cell surface in response to agonist stimulation (Claing et al., 2002), we also examined α_{1D}-AR endocytosis in response to stimulation with the α_1-AR-selective agonist phenylephrine (100 μM). When α_{1D}-AR was expressed alone, stimulation with phenylephrine for 30 min had no significant effect on the small amount of α_{1D}-AR on the cell surface. However, when α_{1D}-AR was coexpressed with β_2-AR and stimulated in the same fashion, more than 40% internalization of α_{1D}-AR was observed. These results indicate that α_{1D}-/β_2-AR heterodimerization not only promotes α_{1D}-AR surface expression, it also enhances α_{1D}-AR-mediated signaling and allows for agonist-promoted internalization of α_{1D}-AR.

Albuterol-Induced Cointernalization and Cross-Desensitization between α_{1D}-/β_2-AR Heterodimers. β_2-AR is known to undergo rapid internalization from the cell surface upon stimulation with β-adrenergic agonists (Claing et al., 2002). Thus, to further assess the potential functional importance of the physical interaction between β_2-AR and α_{1D}-AR, we examined the possibility of cointernalization between these two receptors. As expected, a 30-min treatment with the β_2-AR selective agonist albuterol (10 μM) resulted in more than 40% internalization of β_2-AR, independent of whether the receptor was expressed alone or with α_{1D}-AR (data not shown). When α_{1D}-AR was expressed alone, no change in receptor surface expression was observed upon albuterol stimulation. Interestingly, however, α_{1D}-AR underwent a robust cointernalization (~35%) when coexpressed with β_2-AR and stimulated with albuterol (Fig. 4C). These data suggest that heterodimerization with β_2-AR allows for agonist-promoted cointernalization of α_{1D}-AR.

GPCR internalization is known to play a role in regulating receptor desensitization and resensitization (Claing et al., 2002). Since we found that α_{1D}-AR can undergo cointernalization with agonist-stimulated β_2-AR, we next examined whether α_{1D}-AR signaling might become desensitized upon agonist activation of coexpressed β_2-AR. HEK-293 cells expressing α_{1D}-AR or the α_{1D}-/β_2-AR combination were pretreated for 30 min with 10 μM albuterol, then stimulated with 100 μM NE to examine α_{1D}-AR-induced Ca^{2+} mobilization. Strikingly, pretreatment with 10 μM albuterol resulted in a nearly complete attenuation of the aforementioned large increase in NE-stimulated Ca^{2+} mobilization in cells coexpressing α_{1D}-/β_2-ARs, whereas it had no effect at all in cells expressing α_{1D}-AR alone (Fig. 5). To examine whether this effect might be due to β_2-AR-induced increases in cellular cAMP, we pretreated matched plates of cells with 20 μM forskolin, which directly activates adenylyl cyclase, instead of albuterol. However, no effect of forskolin pretreatment on NE-stimulated Ca^{2+} mobilization was observed (Fig. 5). To assess whether the effects of the albuterol pretreatment might be due to α_{1D}-/β_2-AR cointernalization, we also performed experiments where cells were pretreated with concanavalin A, which prevents receptor internalization (Waldo et al., 1983), prior to albuterol pretreatment. Under these conditions, albuterol pretreatment had no detectable effect on NE-stimulated Ca^{2+} mobilization in cells coexpressing α_{1D}-/β_2-ARs. Taken together, these results suggest that

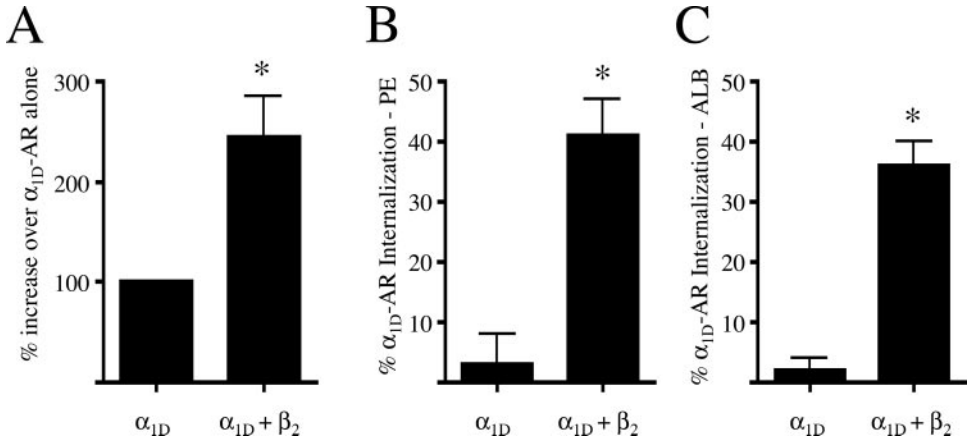


Fig. 4. Coexpression with β_2-AR increases α_{1D}-AR coupling to intracellular Ca^{2+} mobilization and α_{1D}-AR agonist-promoted internalization. A, α_{1D}-AR was expressed either alone or with β_2-AR in HEK-293 cells. The cells were loaded with fura-2, stimulated with 100 μM NE, and analyzed for changes in $[Ca^{2+}]_i$. The values for each experiment are represented as the percentage increase in Ca^{2+} mobilization over α_{1D}-AR expressed alone. B, agonist-induced internalization of α_{1D}-AR in response to phenylephrine. Flag-α_{1D}-AR was expressed either alone or in the presence of HA-β_2-AR in HEK-293 cells, and α_{1D}-AR internalization was examined using a luminometer-based assay following a 30-min stimulation with the α_1-AR-selective agonist, phenylephrine (PE; 100 μM). C, cointernalization of α_{1D}-AR with β_2-AR in response to albuterol. Flag-α_{1D}-AR was expressed either alone or in the presence of HA-β_2-AR in HEK-293 cells, and α_{1D}-AR internalization was examined using a luminometer-based assay following a 30-min stimulation with the selective β_2-AR-selective agonist, albuterol (ALB; 10 μM). For each panel of this figure, * indicates significantly different ($p < 0.05$) relative to α_{1D}-AR expressed alone, as assessed using an unpaired Student's t test analysis. The bars and error bars represent the means ± S.E.M. for three to four independent experiments, with each experiment performed in triplicate.

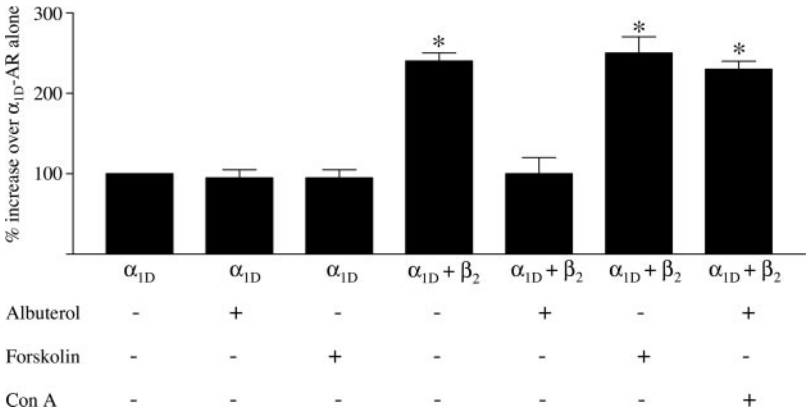


Fig. 5. Cross-desensitization of α_{1D}-AR by β_2-AR is dependent on receptor internalization. Cells were pretreated with albuterol alone (10 μM for 30 min), forskolin alone (20 μM for 30 min), or concanavalin A (10 μM for 15 min) followed by albuterol (10 μM for 30 min), as indicated. The cells were then rinsed, loaded with fura-2, stimulated with 100 μM NE, and analyzed for changes in $[Ca^{2+}]_i$. The values for each experiment are represented as a percentage of the amount of Ca^{2+} mobilization observed for α_{1D}-AR expressed alone. Albuterol pretreatment almost completely blocked α_{1D}-AR-mediated Ca^{2+} mobilization in cells coexpressing β_2-AR, revealing cross-desensitization between β_2-AR and α_{1D}-AR. This effect was not mimicked by forskolin but was blocked by concanavalin A, suggesting a dependence on the cointernalization of α_{1D}-AR with β_2-AR. The data are expressed as mean ± S.E.M. for three to four individual experiments. *, significantly different ($p < 0.05$) relative to nonpretreated α_{1D}-AR alone, as assessed using an unpaired Student's t test analysis.

α_{1D}-AR signaling can be desensitized via cointernalization with β_2-AR.

Discussion

The α_{1D}-AR has been an enigma in the adrenergic field for many years due to the fact that it is inefficiently trafficked to the plasma membrane and, therefore, very difficult to study when heterologously expressed in most cell types (Theroux et al., 1996; Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002; Hague et al., 2004b). We have previously shown that α_{1D}-AR coexpression with α_{1B}-AR results in markedly enhanced trafficking to the plasma membrane (Uberti et al., 2003; Hague et al., 2004b). Here, we show that coexpression with β_2-AR, another AR family member, is also capable of dramatically increasing α_{1D}-AR surface expression in heterologous cells. Importantly, the additional α_{1D}-ARs that make it to the cell surface due to heterodimerization with β_2-AR are functional since we observed significantly enhanced NE-stimulated Ca^{2+} mobilization in cells cotransfected with β_2-AR. These findings provide an additional mechanism for the efficient trafficking of functional α_{1D}-AR to the cell surface beyond heterodimerization with α_{1B}-AR, which is important because mice lacking α_{1B}-AR are known to retain at least some α_{1D}-AR-mediated responses in certain tissues (Cavalli et al., 1997; Turnbull et al., 2003).

Our screen with approximately 30 different group I GPCRs demonstrated that α_{1D}-AR heterodimerization is extremely selective. Notably, we found that β_2-AR, but not the closely related β_1-AR, facilitates α_{1D}-AR surface expression and can be robustly coimmunoprecipitated with α_{1D}-AR from cells. This subtype selectivity parallels our previous findings that α_{1B}-AR, but not the closely related α_{1A}-AR, promotes α_{1D}-AR surface expression and exhibits coimmunoprecipitation with α_{1D}-AR (Uberti et al., 2003; Hague et al., 2004b). Similar observations have recently been made for the specificity of $GABA_BR1$ heterodimerization, with extensive screens revealing that $GABA_BR2$, but not a number of other GPCRs, selectively promotes the surface expression of $GABA_BR1$ (Balasubramanian et al., 2004). Furthermore, subtype-selective heterodimerization has also been observed between certain combinations of other GPCRs (Angers et al., 2002). Such observations belie the notion that GPCR heterodimerization is a nonselective and/or artificial process and suggest instead that these interactions between receptors are quite specific, with this selectivity possibly providing important clues as to the physiological importance of the various associations.

We consistently observed in our studies a small amount of α_{1D}-AR on the cell surface even in the absence of coexpression of either α_{1B}-AR or β_2-AR. This observation might seem to be inconsistent with the idea that α_{1D}-AR requires heterodimerization with other receptors for trafficking to the plasma membrane. However, it is important to point out that HEK-293 cells are known to express a low level of endogenous β_2-AR (Daaka et al., 1997). Thus, in HEK-293 cells transfected with α_{1D}-AR alone, some of the receptor might be able to access the cell surface via heterodimerization with the low level of endogenous β_2-AR, an effect that would presumably be greatly magnified upon β_2-AR overexpression. Such a dependence on heterodimerization with endogenously expressed receptors may help to explain the variability in α_{1D}-AR surface expression that has been observed in different cell types, with transfected α_{1D}-AR in some cells being found almost completely inside the cell in a nonfunctional state (Theroux et al., 1996; McCune et al., 2000; Chalothorn et al., 2002; Hague et al., 2004b), while being found more significantly at the cell surface and more detectably functional in other cell types (Garcia-Sainz and Torres-Padilla, 1999; Garcia-Sainz et al., 2001; Waldrop et al., 2002). The relative levels of α_{1D}-AR-interacting GPCRs such as α_{1B}-AR and β_2-AR expressed in these various cell types may be a central factor in determining the functional activity of transfected α_{1D}-AR.

α_{1D}-AR and β_2-AR are both activated by the same endogenous ligands and are also known to be colocalized in many of the same cells in the cardiovascular, central nervous, and immune systems (Young et al., 1990; Nicholas et al., 1996; Guimaraes and Moura, 2001; Kavelaars, 2002). Moreover, there is an extensive literature on cross talk between α_1- and β-ARs (Akhter et al., 1997; Michelotti et al., 2000; Dzimiri, 2002; Yue et al., 2004). The interaction that we observed in this study between α_{1D}-AR and β_2-AR could serve as a mechanism by which these receptors regulate each other's function in native tissues. Indeed, our studies in heterologous cells demonstrate that a β_2-AR-selective agonist can promote robust cointernalization of α_{1D}-AR. In addition, α_{1D}-AR-mediated Ca^{2+} mobilization was greatly attenuated via pretreatment of cells with a β_2-AR-selective agonist. This effect was probably due to cointernalization of α_{1D}-AR with β_2-AR since it was blocked by concanavalin A pretreatment and was not mimicked by forskolin pretreatment. Taken together, these studies indicate that β_2-AR not only can regulate α_{1D}-AR surface expression but can also control α_{1D}-AR inter-

nalization and desensitization, thereby providing a novel mechanism of cross talk between α_1- and β-ARs.

Like α_{1D}-AR, many other GPCRs, such as α_{2C}-AR (Daunt et al., 1997; Olli-Lahdesmaki et al., 1999; Hurt et al., 2000), $GABA_BR1$ (Marshall et al., 1999; Balasubramanian et al., 2004), trace amine receptors (Bunzow et al., 2001), and odorant receptors (McClintock et al., 1997; Lu et al., 2003; Hague et al., 2004a), among others, are retained in intracellular compartments and, therefore, mostly nonfunctional when expressed alone in heterologous cells. Thus, it is of tremendous physiological importance to understand the mechanisms involved in determining GPCR trafficking to the cell surface. Our studies reveal that subtype-selective heterodimerization is a critical determinant of α_{1D}-AR cell surface expression. Moreover, these studies also provide novel insights into the mechanisms of cross talk between different subfamilies of adrenergic receptor.

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