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ALPHA-2C-ADRENERGIC RECEPTORS EXHIBIT ENHANCED SURFACE EXPRESSION AND SIGNALING UPON ASSOCIATION WITH BETA-2-ADRENERGIC RECEPTORS

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Running title: α_{2C}- and β_2-adrenergic receptor association

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Abbreviations: AR, adrenergic receptor; BSA, bovine serum albumin; DHA, dihydroalprenolol; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal regulated kinase; GPCR, G protein-coupled receptor; HA, Hemagglutinin; HEK, human embryonic kidney; HRP, horse radish peroxidase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RIPA, radio immunoprecipitation assay; SDS, sodium dodecyl sulfate; RX-821002, 2-methoxyidazoxan; UK 14,304, brimonidine.

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ABSTRACT

The alpha-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). Co-transfection of α_{2C}AR with more than twenty-five related GPCRs revealed that only co-expression with the beta-2-adrenergic receptor (β_2AR) increased the surface localization of α_{2C}AR in HEK-293 cells. Co-immunoprecipitation of α_{2C}AR with β_2AR 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 co-expressed with β_2AR was predominantly localized to the plasma membrane. Ligand binding studies revealed a significant increase in α_{2C}AR binding sites upon co-expression with β_2AR, with no apparent change in affinity for α_2AR ligands. Functional assays with the α_2AR-specific agonist UK 14,304 revealed that co-expression of β_2AR with α_{2C}AR enhanced α_{2C}AR-mediated activation of ERK 1/2. Furthermore, analyses of agonist-promoted receptor endocytosis demonstrated enhanced α_{2C}AR internalization in response to α_2AR agonists when α_{2C}AR and β_2AR were co-expressed. Additionally, substantial co-internalization of α_{2C}AR in response to βAR agonists was observed when α_{2C}AR was co-expressed with β_2AR. These data reveal that α_{2C}AR can interact with β_2AR in cells in a manner that regulates α_{2C}AR surface expression, internalization, and functionality.

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INTRODUCTION

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 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 co-expression with α_{1B}AR or β_2AR, presumably due to receptor heterodimerization (Uberti et al., 2003; Hague et al., 2004b; Uberti et al., 2005).

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 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 appear to be limited to homodimers, as 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; Uberti et al., 2005; Hague et al., 2006). In this study, we investigated whether co-expression with other GPCRs might enhance the surface expression and functionality of α_{2C}AR.

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METHODS

Receptor Constructs—$GABA_BR2$ was kindly provided by Fiona Marshall (GlaxoSmithKline). β_1- and β_2-adrenergic receptor constructs were kindly provided by Robert Lefkowitz (Duke University Medical Center). α_{1A}-, α_{1B}-, and α_{1D}-adrenergic receptor constructs were kindly provided by Ken Minneman (Emory University School of Medicine). α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptor constructs were kindly provided by Lee Limbird (Vanderbilt University Medical Center). The β_3-adrenergic receptor was kindly provided by Sheila Collins (CIIT Centers for Health Research). The serotonin 5HT1A receptor construct was kindly provided by John Raymond (Medical University of South Carolina). Angiotensin AT1 and AT2 receptor constructs, trace amine receptors constructs (1-5), $P2Y_2$ receptor construct, NPY1 receptor construct, and thromboxane A2 receptor construct were purchased from the UMR cDNA Resource Center. Muscarinic m1–5 acetylcholine receptor constructs were kindly provided by Allan Levey (Emory University School of Medicine). The $P2Y_1$ receptor construct was kindly provided by Ken Harden (University of North Carolina, Chapel Hill). Opioid receptor constructs (μ, δ, and κ) were kindly provided by Ping-Yee Law (University of Minnesota Medical School). The histamine H3 receptor construct was kindly provided by Tim Lovenberg (The R. W. Johnson Pharmaceutical Research Institute).

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 PCR using the primers GACTCTAGAGCGTCCCCAGCGCTG (5' end, containing the XbaI restriction site) and GTCGGATCCTCACTGCCTGAAGCC (3' end, containing the BamHI restriction site preceded by a stop codon). Following PCR amplification, the receptor and plasmid pDoubleTrouble (pDT), 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 medium 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% CO_2 incubator. To express receptors, 2 μg of DNA from each construct was mixed with LipofectAMINE 2000 (15 μl; Invitrogen, Carlsbad, CA) and added to 5 ml of complete medium in 10-cm tissue culture plates containing cells at ~80–90% confluency. Following over-night incubation, complete medium was added to the culture dishes and cells were trypsinized and re-plated on an appropriately-sized dish.

For confocal microscopy, a transfection efficiency of >80% was achieved (by transfection) using the Nucleofector® and following the protocol supplied by the manufacturer (Amaxa, Gaithersburg, MD). Briefly, HEK-293 cells were trypsinized, collected by centrifugation, and resuspended in Nucleofector solution along with 1 μg 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 18-24 hr.

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, 5% w/v nonfat dry milk, pH 7.5) and incubated with HRP-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 sec 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 DMEM for 30 min at 37°C 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 UK 14,304 (10 μM) or isoproterenol (10 μM) 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 (1x PBS, 2% BSA, 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 Labs, Burlingame, CA). Cells were scanned with a Zeiss LSM 510 laser scanning confocal microscope (Heidelberg, Germany). For detecting Alexa488, fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected for 510-520 nm. For detecting Texas Red, rhodamine fluorescence was excited using a helium-neon laser at a wavelength of 522 nm.

Western blotting—Samples in 1x sample buffer were centrifuged briefly before loading approximately 20 μl of the sample. The proteins were resolved by SDS-PAGE on a 4-20% Tris-Glycine gel, and transferred to PVDF membrane (Millipore). The membranes was incubated for 30 min in Tris-buffered saline with 0.1% Tween-20 (TBS-T) plus 5% dry milk and then with the

appropriate primary antibody for 1 hr. 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 DMEM overnight and exposed to vehicle in the presence or absence of 10 μM UK 14,304 for 5 min at 37°C, added directly to the starvation medium. At the end of the stimulation, the media containing the agent was removed and 60 μl of 1x sample buffer was added. Samples were sonicated, boiled for 5 min, and centrifuged briefly at 17,000 x g before loading 20 μl of each sample. The proteins were resolved by SDS-PAGE, as described above, and the proteins 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 -rabbit were then used for detection by scanning using the Odyssey imaging system, and band density was quantified using Odyssey imaging software (Li-Cor, Lincoln, NE).

Co-immunoprecipitation—Membranes of cells transiently transfected were washed and collected in ice-cold RIPA buffer (50 mM Tris-HCL, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing COMPLETE® protease inhibitor cocktail (Roche, Indianapolis, IN) and incubated for 60 minutes at 4°C with rotation. Unsolubilized membranes were pelleted and the supernatant incubated with anti-Flag conjugated agarose beads overnight at 4°C with rotation. The beads were washed in PBS and the protein eluted from the beads in 1x sample buffer. Samples were analyzed by Western blotting as described above.

Radioligand binding assays—Cells were washed, collected, and centrifuged at 50,000 x g to collect the membranes, sonicated briefly and resuspended in 3 ml of fresh binding buffer. The

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affinity of the receptors for [^{3}H]DHA (β_2AR antagonist) or [^{3}H]rauwolscine (α_{2C}AR antagonist) was assessed in saturation binding assays using six concentrations of [^{3}H]DHA or [^{3}H]rauwolscine. The membrane preparation was incubated with [^{3}H]DHA or [^{3}H]rauwolscine for 30 min at 22°. The reaction was stopped by filtration through Whatman GF/C glass fiber filters on a Brandel cell harvester. The amount of [^{3}H] ligand present was determined by liquid scintillation counting. Non-specific binding was defined using 10 mM propranolol for β_2AR or 10 mM norepinephrine or RX 821002 for α_{2C}AR. Nonlinear regression analyses of saturation binding assays and statistical comparisons were performed with GraphPad Prism (GraphPad Software).

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RESULTS

Localization of α_{2C}AR following co-transfection with other GPCRs. In order to investigate the effect of heterodimerization on α_{2C}AR surface expression, α_{2C}AR was co-expressed with a panel of 29 different GPCRs. The relative increase in Flag-tagged α_{2C}AR surface expression was investigated using an intact-cell ELISA assay that has been utilized previously to study other trafficking-defective GPCRs (Uberti et al., 2003; Hague et al., 2004a; Hague et al., 2004b; Uberti et al., 2005). Co-expression with most of the receptors examined had no detectable effect on the localization of α_{2C}AR, but co-transfection with β_2AR caused a marked increase (four-fold) in the amount of α_{2C}AR at the cell surface (Figure 1).

The effect of β_2AR on α_{2C}AR localization was confirmed via a second and independent technique, confocal microscopy. As described previously (von Zastrow et al., 1993; Daunt et al., 1997), α_{2C}AR expressed alone in HEK-293 cells is largely intracellular (Figure 2A). In contrast, β_2AR expressed alone in our studies displayed strong surface localization in HEK-293 cells (Figure 2B). Upon co-expression of β_2AR with α_{2C}AR, β_2AR localization was unaltered by co-expression with α_{2C}AR, but there was a striking increase in the surface localization of α_{2C}AR, such that α_{2C}AR colocalized well with β_2AR at the plasma membrane (Figure 2C-E).

One possible explanation for the ability of β_2AR to alter the trafficking of α_{2C}AR is an interaction between the two receptors. To explore this possibility, we investigated the ability of α_{2C}AR to interact with β_2AR by co-immunoprecipitation. Immunoreactivity for Flag-α_{2C}AR was evident as a major band at ~45 kDa and a second band at approximately 100 kDa, which may represent receptor multimers not fully resolved on SDS-PAGE. Both α_{2C}AR bands were efficiently immunoprecipitated with anti-Flag antibodies (Figure 3). The major band of HA-β_2AR immunoreactivity (~52 kDa) was not immunoprecipitated by anti-Flag antibodies when

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β_2AR was expressed alone. However, HA-β_2AR was robustly co-immunoprecipitated with Flag-α_{2C}AR when the two receptors were expressed together. These data reveal that α_{2C}AR and β_2AR can form a complex in a cellular environment.

Binding properties of α_{2C}AR and β_2AR. The effects of receptor co-expression on binding affinity and total receptor number for α_{2C}AR and β_2AR were assessed in saturation binding assays. Using the α_2AR-specific ligand rauwolscine, we observed that the K_D value was unchanged by co-expression with β_2AR, but the B_{max} value was increased by approximately 2-fold. Conversely, neither the K_D nor B_{max} values for [^{3}H]dihydroalprenolol (DHA) binding were altered when β_2AR was co-expressed with α_{2C}AR (Figure 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_{2C}AR or β_2AR to compete with their respective radioligands. However, competition binding assays revealed that the affinity values for UK 14,304 and norepinephrine binding to α_{2C}AR were not significantly different when α_{2C}AR was expressed alone versus co-expressed with β_2AR (Figure 5). The affinity values for epinephrine and isoproterenol binding to β_2AR were also not changed when β_2AR was co-expressed with α_{2C}AR (Table 1).

Effect of α_{2C}AR/β_2AR co-expression on α_{2C}AR functionality. The α_2AR 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. We therefore explored the effect of β_2AR co-expression on the functional properties of α_{2C}AR using the phosphorylation of ERK1/2 as an endpoint. Cells transfected with α_{2C}AR in the absence or presence of β_2AR were incubated with UK 14,304. Increases in ERK1/2

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phosphorylation were observed in both cases. However, the observed increase in ERK1/2 phosphorylation was much larger when α_{2C}AR was co-expressed with β_2AR than when α_{2C}AR was expressed alone. The stimulatory effects of UK 14,304 in all cases were blocked by RX-821002, an α_2AR antagonist (Figure 6). In contrast to the large effect of β_2AR co-expression on α_{2C}AR signaling, β_2AR-mediated stimulation of ERK1/2 phosphorylation by isoproterenol was not significantly altered by co-expression with α_{2C}AR (data not shown).

The predominantly intracellular localization of α_{2C}AR in most cell types has been a confounding factor in previous studies aimed at assessing the capacity of α_{2C}AR to undergo agonist-promoted endocytosis (Daunt et al., 1997; DeGraff et al., 1999; Olli-Lahdesmaki et al., 1999). However, the ability of β_2AR to traffic α_{2C}AR to the plasma membrane enabled us to more easily investigate α_{2C}AR internalization following agonist stimulation. When α_{2C}AR was expressed alone and stimulated with UK 14,304, the small population of α_{2C}ARs on the cell surface did not undergo any significant internalization, as assessed using the luminometer-based whole-cell ELISA assay. When α_{2C}AR was co-expressed with β_2AR, however, there was a striking 30% decrease in the amount of α_{2C}AR on the cell surface following a 30-minute treatment with UK 14,304. Further, the β_2AR-specific agonist isoproterenol also resulted in substantial endocytosis of α_{2C}AR, suggesting cross-internalization between the two receptors (Figure 7A).

The effect of co-expression with α_{2C}AR on β_2AR internalization was also examined. As expected, a 30-minute treatment with isoproterenol caused a robust 35% β_2AR endocytosis, and this isoproterenol-induced internalization was not altered by co-expression of α_{2C}AR. Unlike the apparent cross-internalization of α_{2C}AR following isoproterenol stimulation of co-expressed

β_2AR, UK 14,304 stimulation of α_{2C}AR was unable to promote internalization of co-expressed β_2AR (Figure 7B).

Agonist-induced receptor internalization was also studied via confocal microscopy. When α_{2C}AR and β_2AR were co-expressed 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 (Figure 8). In contrast, stimulation of the doubly-transfected cells with UK 14,304 resulted in endocytosis of α_{2C}AR but not β_2AR (data not shown). Thus the data from the confocal studies matched well the results from the luminometer-based assay described above, in that both techniques revealed co-internalization of the α_{2C}AR/β_2AR complex upon treatment with βAR agonists.

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DISCUSSION

A number of GPCRs, including $GABA_BR1$, $\alpha_{1D}AR$, $\alpha_{2C}AR$, 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 co-expression with $GABA_BR2$ can facilitate $GABA_BR1$ trafficking to the cell surface suggested a key role for receptor heterodimerization in regulating the trafficking of certain GPCRs (Marshall et al., 1999). Similarly, associations of $\alpha_{1D}AR$ and olfactory receptors with specific GPCR partners have been found to enhance the surface expression of these trafficking-defective receptors (Uberti et al., 2003; Hague et al., 2004a; Hague et al., 2004b; Uberti et al., 2005). The purpose of the studies reported here was to investigate whether the poor trafficking of $\alpha_{2C}AR$ might also be enhanced by co-expression with an appropriate GPCR partner.

After examining $\alpha_{2C}AR$ surface trafficking following co-expression with more than 25 different GPCRs, we observed that surface expression of $\alpha_{2C}AR$ was markedly enhanced only by co-expression with β_2AR. Confocal microscopy studies confirmed increased surface expression of $\alpha_{2C}AR$ upon β_2AR co-expression. We also observed that β_2AR could be robustly co-immunoprecipitated with $\alpha_{2C}AR$. Thus, a reasonable interpretation of these data is that $\alpha_{2C}AR$ surface expression is enhanced via association with β_2AR, although it is not entirely clear if the $\alpha_{2C}AR/\beta_2AR$ interaction is direct (via heterodimerization) or indirect (via joint interaction with a scaffold protein). In any case, the effects of β_2AR co-expression on $\alpha_{2C}AR$ surface trafficking are analogous to previous observations that interactions with either $\alpha_{1B}AR$ or β_2AR enable $\alpha_{1D}AR$ to localize normally to the plasma membrane (Uberti et al., 2003; Hague et al., 2004b; Uberti et al., 2005). The effects of receptor co-expression on the trafficking of both $\alpha_{2C}AR$ and $\alpha_{1D}AR$ seem to be quite specific, since the vast majority of receptors examined had

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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 3 cloned opioid receptors expressed by themselves (Jordan and Devi, 1999; George et al., 2000). In our studies, co-expressed α_{2C}AR and β_2AR 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 [^{3}H]rauwolscine binding was observed in saturation binding assays, where α_{2C}AR levels were increased by almost 2-fold when co-expressed with β_2AR, and a similar increase was also observed for α_{2C}AR immunoreactivity upon β_2AR co-expression (data not shown). Increased receptor stability has been described for other trafficking-defective receptors upon co-expression with appropriate partners, such as α_{1D}AR co-expressed with α_{1B}AR (Uberti et al., 2003). The observed increases in α_{2C}AR levels upon β_2AR co-expression might be explained by reduced α_{2C}AR retention in the endoplasmic reticulum, where accumulating α_{2C}AR would be rapidly degraded. Thus, since association with β_2AR 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.

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Receptor-receptor interactions are known to have strong effects on regulating signaling for certain GPCR combinations. In the case of trafficking-defective GPCRs like 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 co-expression with β_2AR. 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_2AR co-expression, was fully blocked by pertussis toxin treatment (data not shown), suggesting predominant coupling of α_{2C}AR to $G_{i/o}$ even following association with β_2AR. Thus, since α_{2C}AR ligand binding and G protein coupling specificity did not seem to be altered by co-expression with β_2AR, the most plausible explanation for the enhanced signaling is that β_2AR-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. While α_{2C}AR is largely intracellular and non-functional 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_2AR 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

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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). Since the retention of misfolded proteins by the ER/Golgi complex is known to be less efficient at lower temperatures (Morello et al., 2000), it seems likely that an impairment in the cells' ability to retain α_{2C}AR accounts for the reported effect of temperature on α_{2C}AR trafficking. While 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 cellular 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 MDCK 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 over-expressed (DeGraff et al., 1999). Since β_2AR co-transfection 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. Further, because α_{2C}AR and β_2AR 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 co-expressed with β_2AR. We also observed a marked internalization of α_{2C}AR in response to

isoproterenol, indicating that α_{2C}AR undergoes co-internalization with β_2AR upon β_2AR agonist stimulation. These findings were confirmed by confocal microscopy studies, which showed co-localization of α_{2C}AR and β_2AR in intracellular punctate regions following stimulation with isoproterenol. Interestingly, as with the luminometer assays, internalization of β_2AR did not seem to be affected by UK 14,304 treatment, which may indicate that recruitment of arrestin to the α_{2C}AR/β_2AR complex is dependent on whether the α_{2C}AR component or β_2AR component is stimulated by agonist. The isoproterenol-stimulated internalization of α_{2C}AR observed here suggests a mechanism that may underlie various forms of cross-talk that have been reported between β_2ARs and α_2ARs (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_2AR are co-expressed 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_2AR associations in native tissues, for example studies on knockout mice, may shed additional light on the physiological importance of the interaction between these receptors *in vivo*.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Co-expression with β_2AR enhances α_{2C}AR surface expression. HEK-293 cells were transfected with α_{2C}AR alone or co-transfected with α_{2C}AR plus other GPCRs. After 48 hr the cells were fixed and Flag-tagged α_{2C}AR was labeled with anti-Flag HRP-conjugated antibody. Relative luminescence was quantified using a luminometer following incubation with ELISA ECL reagent. Where possible, presence of the co-transfected receptors was confirmed by Western blot. Data shown are from 3-6 separate experiments for each condition. Receptor abbreviations: AR-adrenergic receptor; H-histamine receptor; κ-kappa, δ-delta, and μ-mu opioid receptors (ORs); $5HT_{1A}$-serotonin receptor; m-muscarinic receptor; TP-thromboxane A2 receptor; P2Y-purinergic receptor; TAR-trace amine receptor; NPY-neuropeptide Y receptor; AT-angiotensin receptor. *, $p<0.001$

Figure 2. Co-expression of α_{2C}AR with β_2AR alters the subcellular localization of α_{2C}AR. Flag-α_{2C}AR (A; green) and HA-β_2AR (B; red) were expressed alone or together (C-E) in HEK-293 cells and visualized using secondary antibodies coupled to Alexa488 or Alexa546. In the absence of β_2AR, α_{2C}AR was mainly intracellular. However, α_{2C}AR was found predominantly at the cell surface following co-expression with β_2AR (C-E). These data are representative of at least 3 separate experiments for each condition.

Figure 3. Co-immunoprecipitation of α_{2C}AR with β_2AR. Cells were transfected with Flag-α_{2C}AR alone, Flag-α_{2C}AR/HA-β_2AR, or HA-β_2AR alone. The lysates were incubated with anti-Flag-conjugated beads to immunoprecipitate Flag-α_{2C}AR. The immunoprecipitates (shown in

panels C and D) were examined for Flag and HA immunoreactivity. HA-β_2AR was immunoprecipitated by the anti-Flag antibodies only when co-expressed with Flag-α_{2C}AR. Molecular weight standards are indicated by the numbers to the left. This figure is representative of 5 separate experiments.

Figure 4. Co-expression of α_{2C}AR with β_2AR increases α_{2C}AR binding sites. Membranes from cells transiently expressing α_{2C}AR, α_{2C}AR/β_2AR, or β_2AR were prepared and incubated with varying concentrations of [^{3}H]rauwolscine (A) or [^{3}H]dihydroalprenolol (DHA) (B). The affinity of α_{2C}AR for [^{3}H]rauwolscine was not altered in the absence (filled circles) or presence (open circles) of β_2AR, but the B_{max} was increased (see Table 1). Data shown are representative of 3 separate experiments. Both the affinity of β_2AR for [^{3}H]DHA and the B_{max} were similar when β_2AR was expressed in the absence (filled squares) or presence (open squares) of α_{2C}AR. Data shown are representative of 3 separate experiments; in all cases error at each point was less than 15% of the calculated value.

Figure 5. Binding to agonists is not affected by co-expression of α_{2C}AR with β_2AR. Membranes from cells transiently expressing α_{2C}AR alone, α_{2C}AR/β_2AR, or β_2AR alone were prepared and the ability of varying concentrations of α_{2C}AR agonists (UK 14304, norepinephrine) or β_2AR agonists (epinephrine, isoproterenol) to compete for [^{3}H]rauwolscine or [^{3}H]DHA binding sites was investigated. Data shown are the average of 3 separate experiments.

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Figure 6. Enhanced α_{2C}AR signaling upon co-expression with β_2AR. A) HEK-293 cells transfected with α_{2C}AR in the absence or presence of β_2AR were incubated with vehicle, UK 14,304 (10 μM) or UK 14,304 along with RX-821002 (10 μM) for 5 min. Cells were harvested in 1x sample buffer, resolved by SDS-PAGE and blotted for phospho-ERK1/2. B) The phosphorylated ERK1/2 bands from 4 separate experiments were quantified and normalized to total ERK1/2. *, $p<0.05$.

Figure 7. Co-internalization of α_{2C}AR and β_2AR. A) Cells transfected with Flag-α_{2C}AR were incubated with UK 14,304 (10 μM) or isoproterenol (ISO, 10 μM) for 30 min in the presence or absence of co-expression with HA-β_2AR. 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_2AR were incubated with UK 14,304 or ISO for 30 min in the presence or absence of co-expression with Flag-α_{2C}AR. The dishes were placed on ice, washed twice and fixed. Internalization was defined as the loss of HA-β_2AR from the cell surface using the luminometer-based assay. Data shown are from 4 separate experiments. Asterisks indicate significant differences from unstimulated cells. (*, $p<0.05$; **, $p<0.01$).

Figure 8. Confocal microscopy analysis of α_{2C}AR co-internalization with β_2AR. In HEK-293 cells, α_{2C}AR and β_2AR were co-expressed and stimulated with isoproterenol (10μM) for 30 min. Flag-α_{2C}AR (green) and HA-β_2AR (red) were visualized using secondary antibodies coupled to Alexa488 or Alexa546. For comparison to unstimulated cells, please compare these data to Figure 2, panels C-E. The data shown in this figure are representative of 3 separate experiments.

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	[^{3}H]Rau		[^{3}H]DHA		α_2AR agonists		β_2AR agonists	
	(K_D, pM)	Fold B_{max} Increase	(K_D, pM)	Fold B_{max} Increase	UK (K_i, μM)	NE (K_i, μM)	Iso (K_i, μM)	EPI (K_i, μM)
α_{2C}AR	165±18	1.83±0.18	n.d.	n.d.	0.198±.020	2.83±0.120	n.d.	n.d.
α_{2C}AR/β_2AR	179±22		47.2±4.0	0.97±0.16	0.170±.008	2.60±0.250	1.52±0.08	0.473±0.035
β_2AR	n.d.	n.d.	42.6±4.3		n.d.	n.d.	1.28±0.03	0.239±0.005

Table 1. Ligand binding properties of α_{2C}AR and β_2AR expressed separately or in combination. Membranes derived from HEK-293 cells transiently transfected with α_{2C}AR and/or β_2AR were examined in saturation binding assays to determine affinity constants for [^{3}H]rauwolscine (Rau - α_2 antagonist) or [^{3}H]dihydroalprenolol (DHA - β_2AR antagonist). K_i values for α_{2C}AR agonists (UK - UK 14,304, NE - norepinephrine) were determined in competition assays with [^{3}H]Rau, and K_i values for β_2AR agonists (Iso - isoproterenol, Epi - epinephrine) were determined in competition assays with [^{3}H]DHA. Values are mean ± SEM of 3-5 experiments. n.d. - no determination made.

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Figure 1

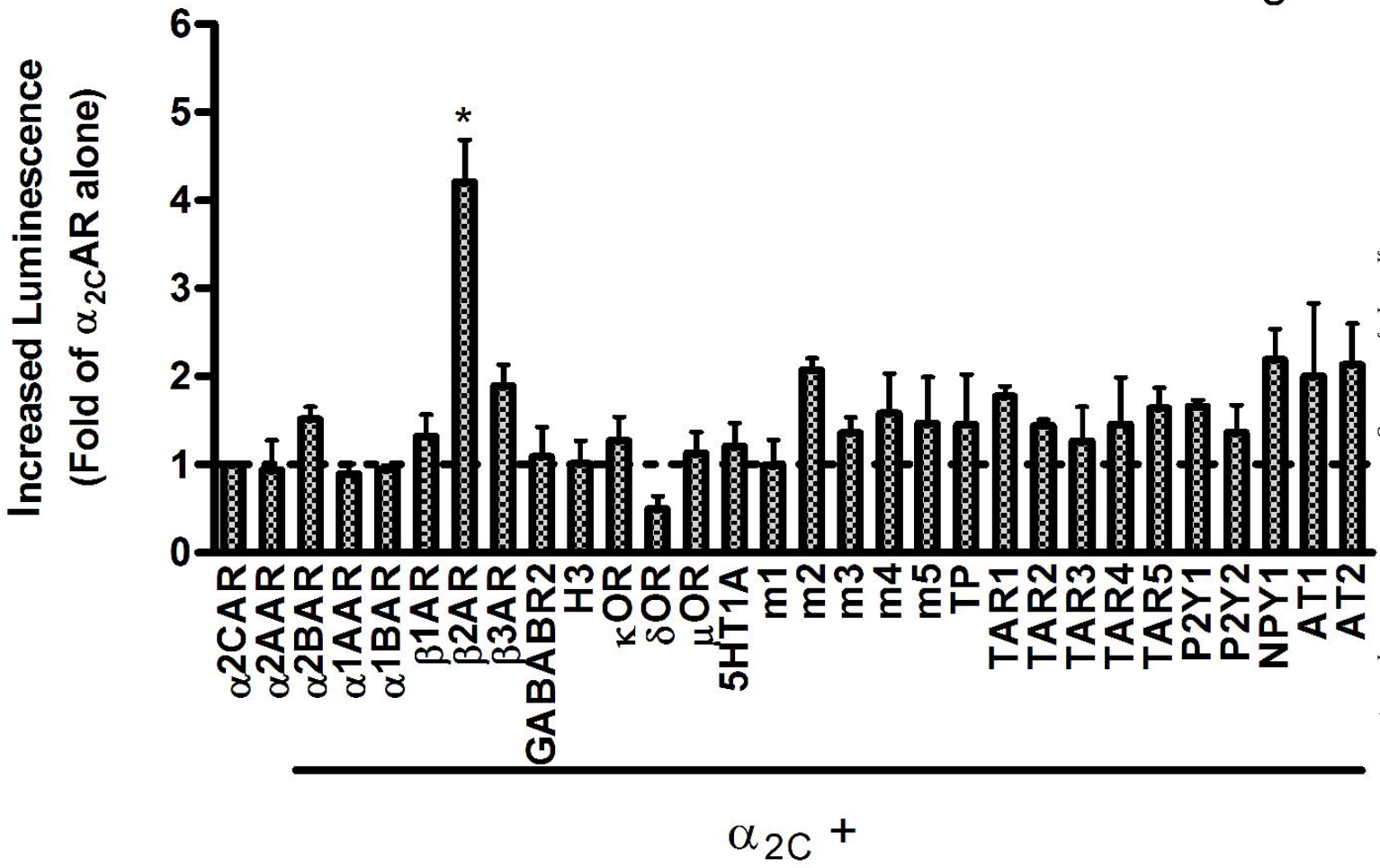

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Figure 2

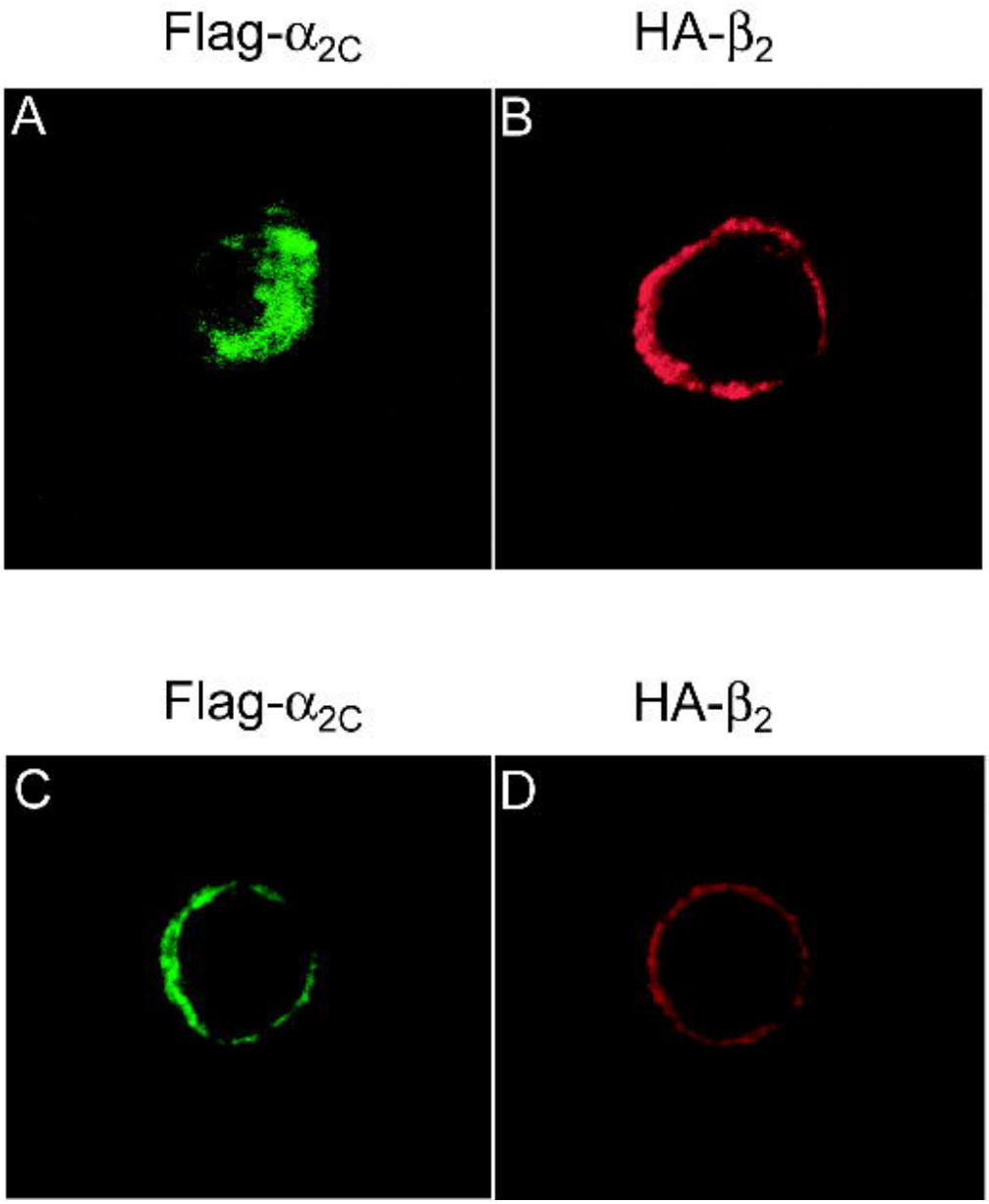

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Figure 3

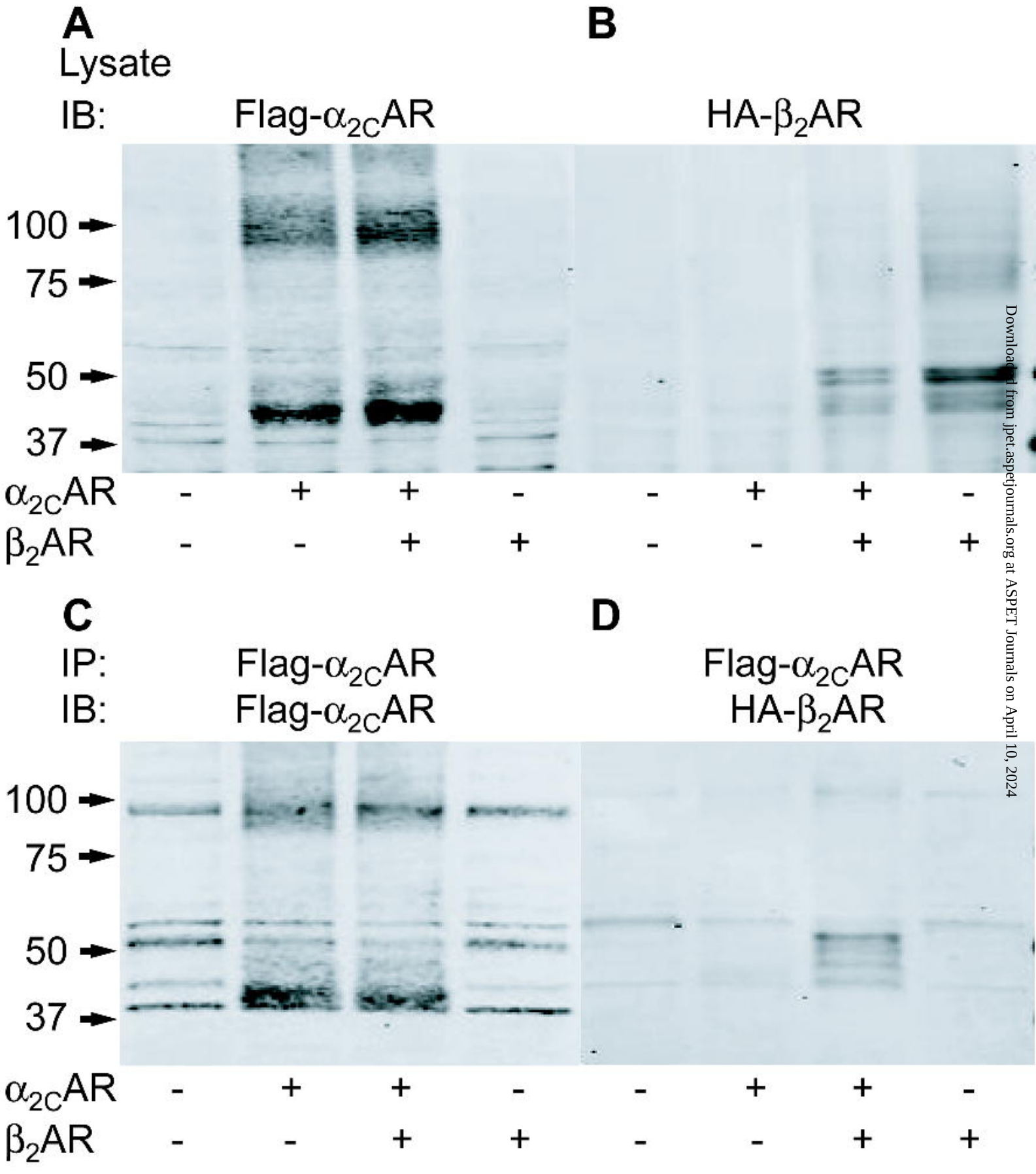

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Figure 4

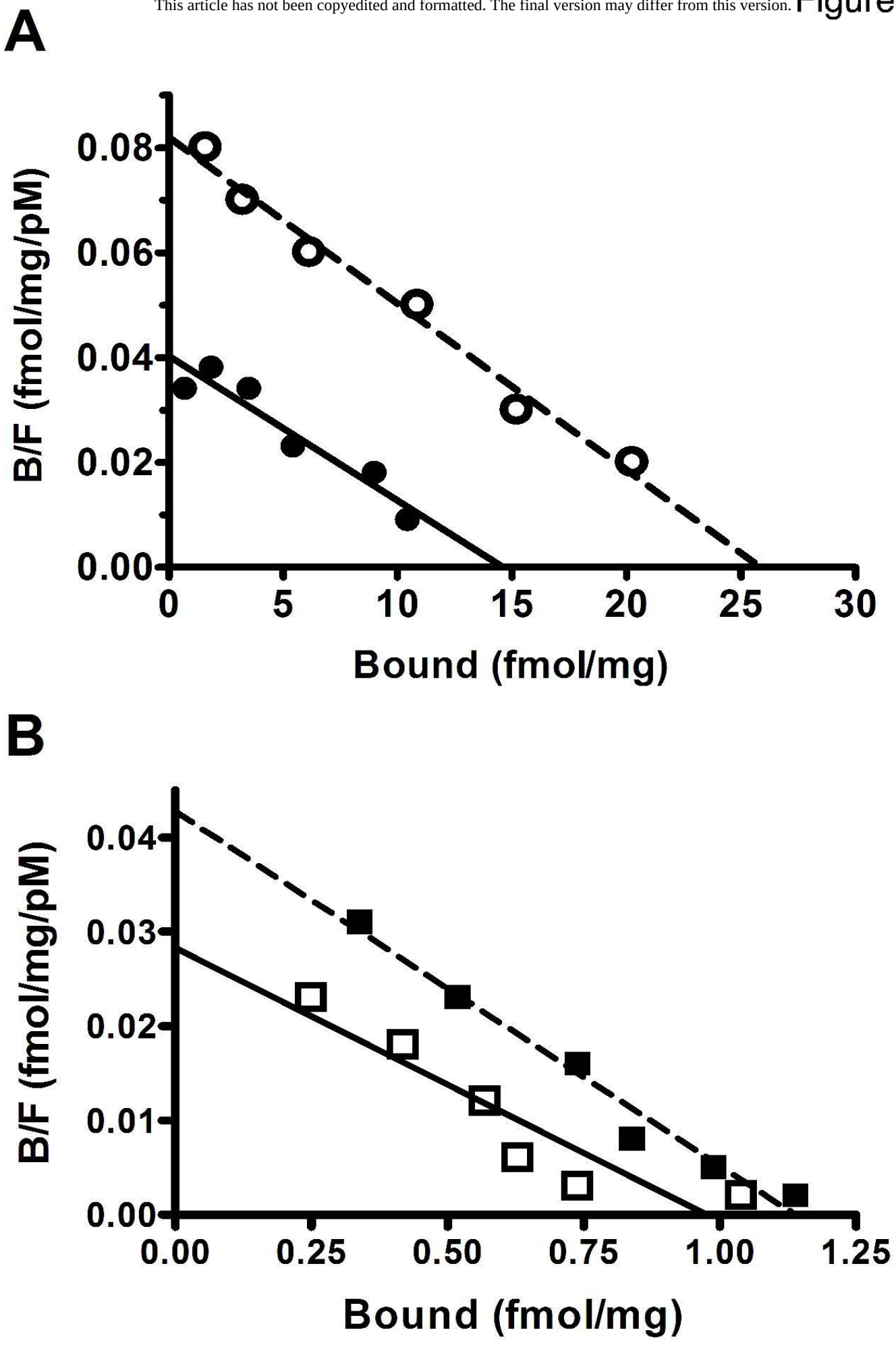

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Figure 5

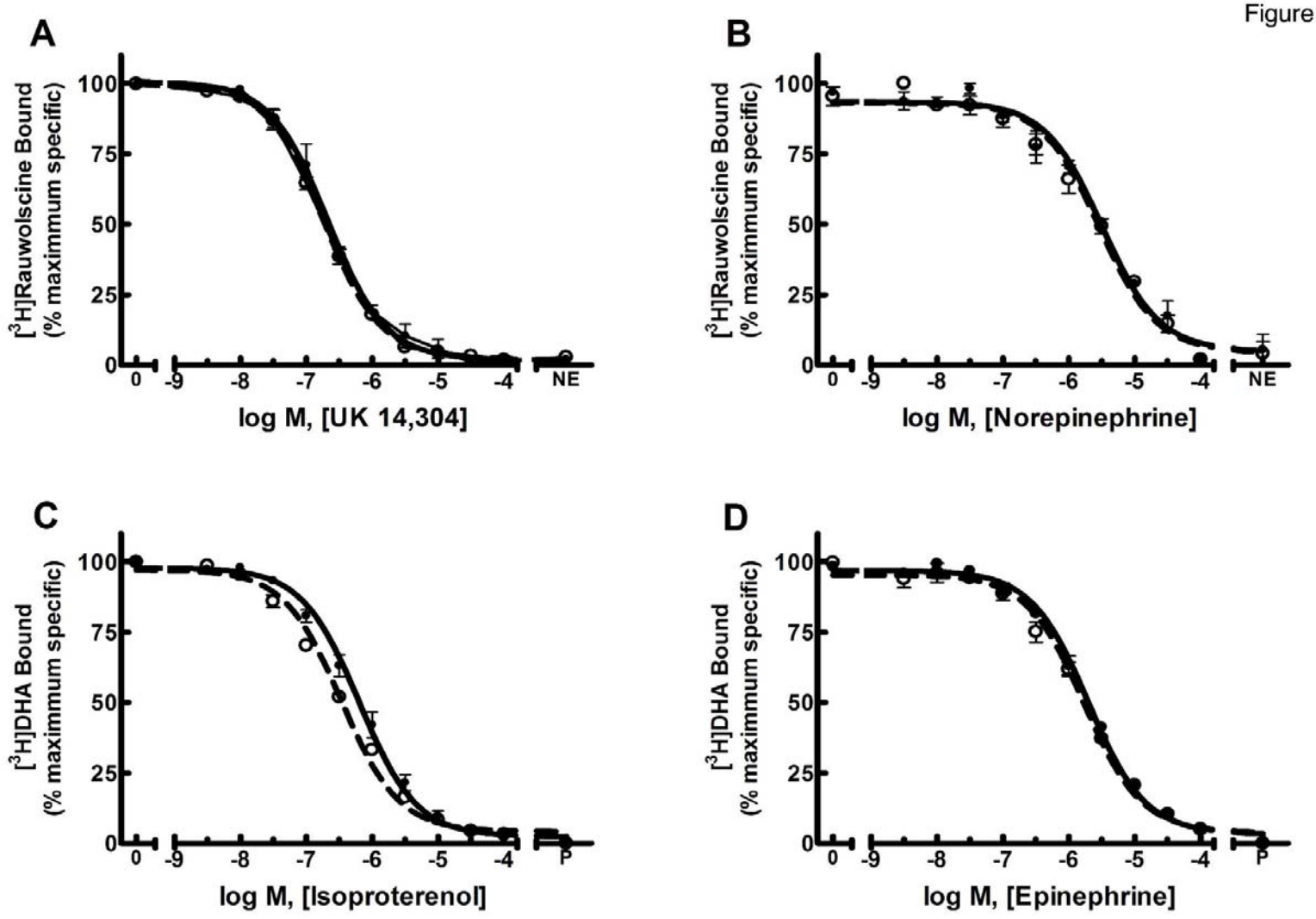

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Figure 6

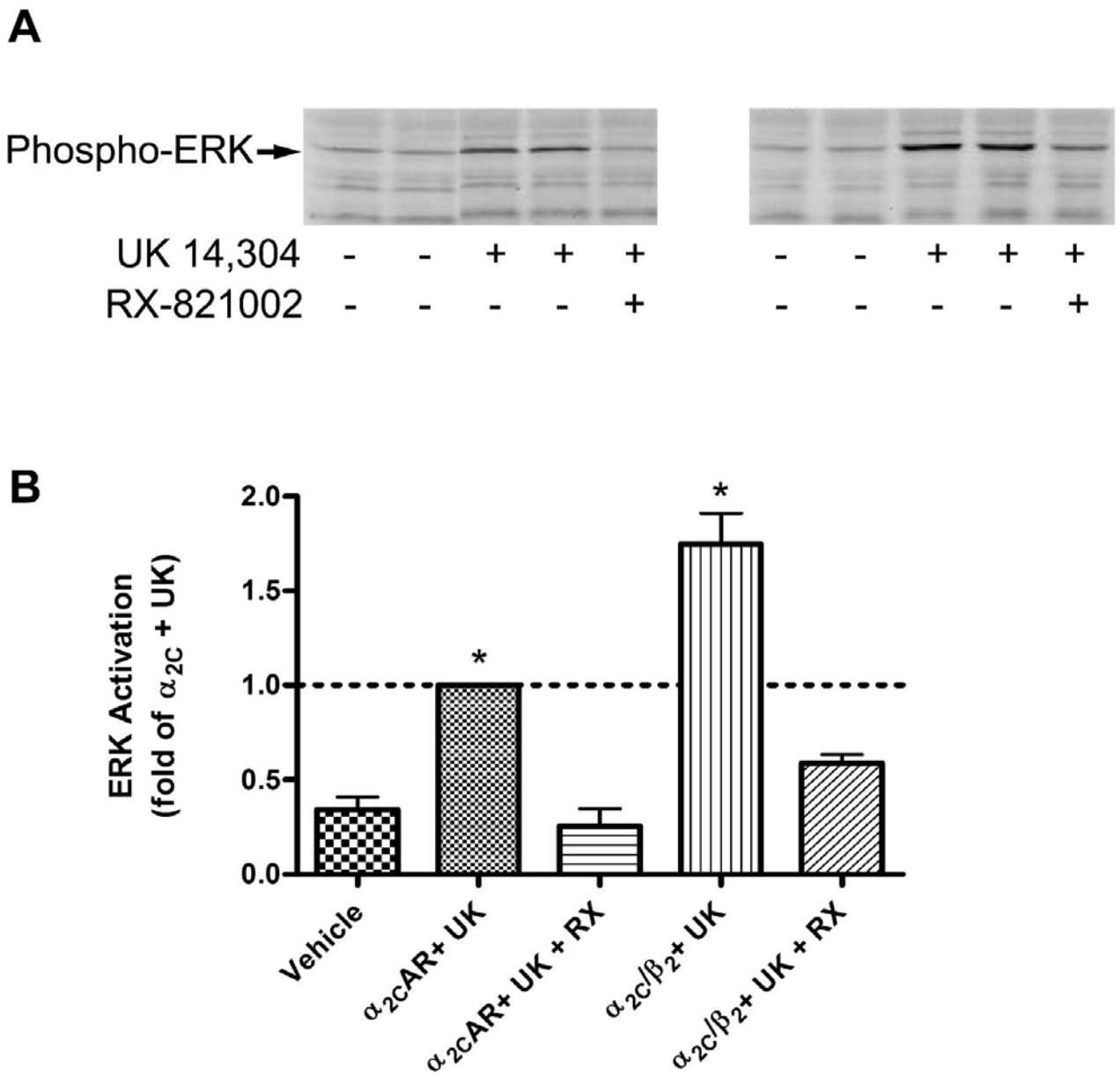

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Figure 7

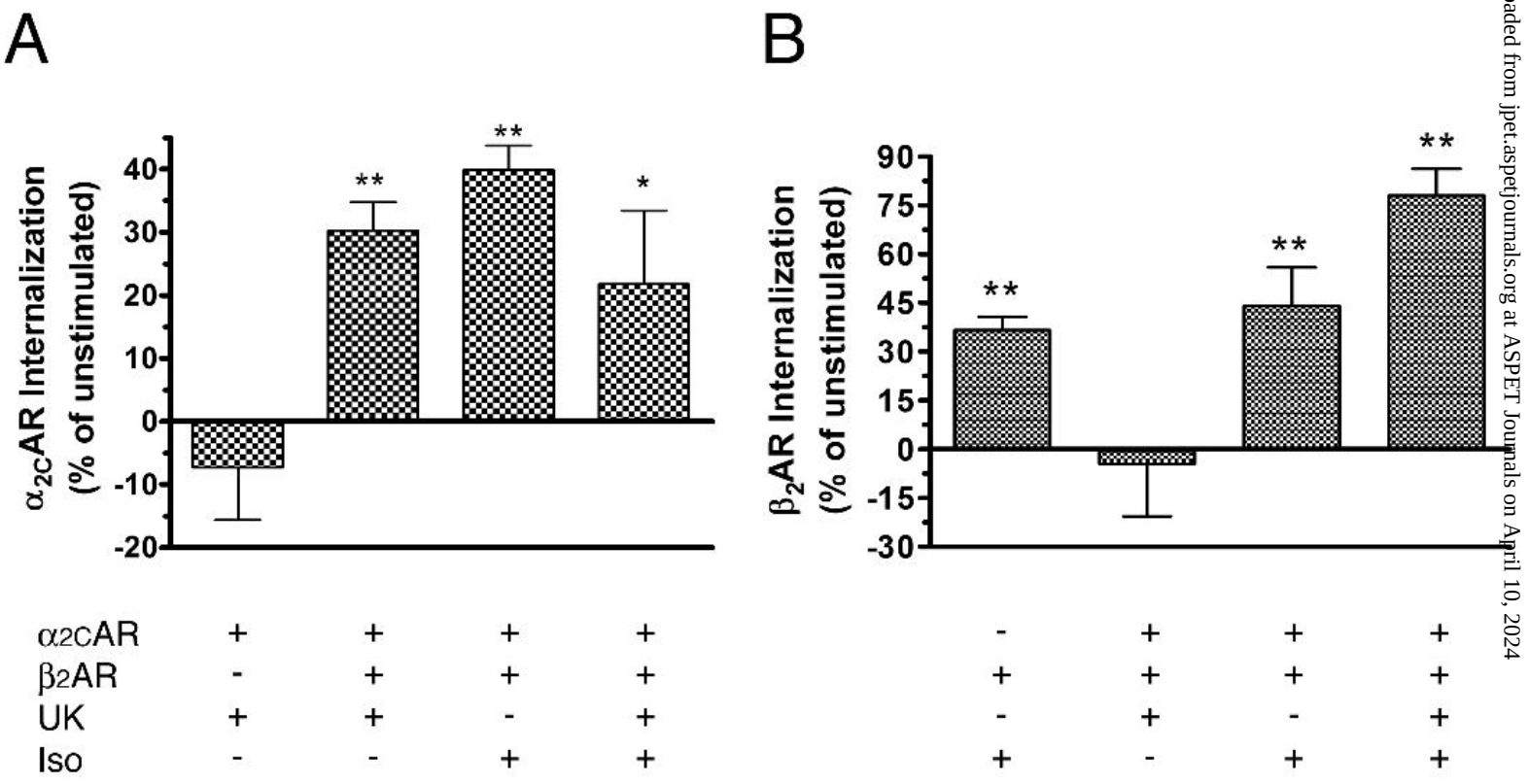

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Figure 8