

# Subtype-Specific Dimerization of $\alpha_1$ -Adrenoceptors: Effects on Receptor Expression and Pharmacological Properties

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Received January 28, 2003; accepted August 29, 2003

This article is available online at <http://molpharm.aspetjournals.org>

## ABSTRACT

The potential role of dimerization in controlling the expression and pharmacological properties of  $\alpha_1$ -adrenoceptor subtypes was examined using coimmunoprecipitation of epitope-tagged receptors. Human  $\alpha_1$ -adrenoceptor subtypes ( $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1D}$ ) were tagged at their amino-termini with Flag or hemagglutinin epitopes and transfected into human embryonic kidney 293 cells. Homodimerization of all three subtypes was observed by coimmunoprecipitation of receptors with different tags and was not altered by norepinephrine treatment. Heterodimer formation between hemagglutinin-tagged  $\alpha_{1B}$ -adrenoceptors and Flag-tagged  $\alpha_{1A}$ - or  $\alpha_{1D}$ -adrenoceptors was also observed. However, no  $\alpha_{1A}/\alpha_{1D}$ -adrenoceptor heterodimers were observed, suggesting that dimerization is subtype-specific. The extent of heterodimerization was also unaltered by norepinephrine treatment.  $\alpha_1$ -Adrenoceptor truncation mutants lacking carboxyl or amino-terminal sequences formed homo- and heterodimers similarly to full-length receptors, suggesting that these domains play little or no role in dimerization. Biotinylation

with a membrane-impermeable agent showed that monomers and homo- and hetero-oligomers of all three subtypes are expressed on the cell surface. Radioligand binding studies showed that heterodimerization did not alter the affinity of  $\alpha_1$ -adrenoceptors for norepinephrine, prazosin, or subtype-selective antagonists, suggesting that dimerization does not result in pharmacologically distinct subtypes. However, coexpression of  $\alpha_{1B}$ -adrenoceptors significantly increased both binding site density and protein expression of  $\alpha_{1A}$ - and  $\alpha_{1D}$ -adrenoceptors, and increased cell surface expression of  $\alpha_{1D}$ -adrenoceptors, suggesting a functional role for heterodimerization. Conversely, coexpression of  $\alpha_{1A}$ -with  $\alpha_{1D}$ -adrenoceptors, which did not heterodimerize, had no effect on receptor density or protein. These studies demonstrate subtype-selective heterodimerization of  $\alpha_1$ -adrenoceptors, which does not change their pharmacological properties but seems to have functional consequences in regulating receptor expression and trafficking.

Dimerization of growth factor and cytokine receptors is essential for their signaling (Heldin, 1995). However, G-protein coupled receptors (GPCRs) have traditionally been thought to function as monomers, with a single receptor binding ligand and activating G-proteins. Early studies, using muscarinic/adrenergic receptor chimeras, suggested that the monomeric GPCR model may not completely explain the mechanisms of GPCR function. These studies demonstrated that two binding-deficient muscarinic/adrenergic chimeras could form a receptor capable of specific ligand binding and signaling upon coexpression. Thus, it was suggested that a direct interaction between the two chimeras could reconstitute a ligand binding pocket (Maggio et al., 1993).

Strong evidence now suggests that some GPCRs, such as GABA<sub>B</sub> and taste receptors, exist as obligate heterodimers and that dimerization may alter the functional, pharmacological or regulatory properties of many other GPCRs (Bouvier, 2001; Rios et al., 2001). These observations have revolutionized contemporary views of GPCR function, raising previously unconsidered possibilities of multiple subunits, multiple binding sites, and additional subtypes that cannot be explained by existing GPCR clones expressed in isolation. GPCR dimerization may be mediated by covalent and/or non-covalent interactions, may involve extracellular, transmembrane, or intracellular domains, and, in some cases, may be altered by agonist occupancy (Bouvier, 2001; Rios et al., 2001). Strikingly, two closely related opioid receptors ( $\kappa$  and  $\delta$ ) heterodimerize to form receptors with distinct ligand binding profiles and functional properties (Jordan and Devi, 1999; Jordan et al., 2000). These studies suggest that dimer-

Supported by National Institutes of Health grants to KPM and RAH. RAH thanks the W.M. Keck Foundation for a Distinguished Young Scholar in Medical Research award.

**ABBREVIATIONS:** GPCR, G protein coupled receptor; AR, adrenoceptor; HEK, human embryonic kidney; BMV7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione dihydrochloride; HRP, horseradish peroxidase; HA, hemagglutinin; ECL, enhanced chemiluminescence; D $\beta$ M, *n*-dodecyl- $\beta$ -D-maltoside; PBS, phosphate-buffered saline; PNGase F, *N*-glycosidase F; TBST, Tris-buffered saline containing 0.1% Tween-20; PAGE, polyacrylamide gel electrophoresis; sulfo-LC-NHS-biotin, sulfosuccinimidyl-6-(biotinamido) hexanoate; BE 2254, 2-[ $\beta$ -(4-hydroxyphenyl)-ethylaminomethyl]-tetralone; <sup>125</sup>I-BE, <sup>125</sup>I-BE 2254; NE, norepinephrine.

ization of GPCRs may represent a novel mechanism modulating receptor pharmacology, trafficking and/or function.

$\alpha_1$ -Adrenoceptors ( $\alpha_1$ -ARs) are  $G_{q/11}$  coupled receptors that mediate responses to the neurotransmitters and hormones norepinephrine (NE) and epinephrine.  $\alpha_1$ -ARs initiate signals by activating phospholipase C and generating second messengers that release stored intracellular  $Ca^{2+}$  and stimulate protein kinase C. Three human  $\alpha_1$ -AR subtypes ( $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1D}$ ) have been cloned, each encoded by different genes (Zhong and Minneman, 1999). These subtypes are highly homologous within their transmembrane domains but share little homology at their amino and carboxyl termini (Schwinn et al., 1995). Localization studies have shown that human tissues such as brain, heart, and vascular smooth muscle express mixtures of  $\alpha_1$ -ARs (Rokosh et al., 1996; Zhong and Minneman, 1999), and homogeneous cell populations, such as rat cardiomyocytes and clonal human SK-N-MC neuroepithelioma cells, have also been found to coexpress all three  $\alpha_1$ -AR subtypes (Rokosh et al., 1996; Zhong and Minneman, 1999). Furthermore, there is pharmacological evidence for additional  $\alpha_1$ -AR subtypes that cannot be explained by the existing clones (Endoh, 1996; Ford et al., 1996, 1997; Rokosh et al., 1996; Zhong and Minneman, 1999). For example,  $\alpha_{1L}$ -AR, a subtype that has a relatively low affinity for prazosin, has been described in functional studies but has not been identified molecularly (Ford et al., 1997; Muramatsu et al., 1998). Other studies have also indicated discrepancies between native  $\alpha_1$ -ARs and recombinant subtypes (Johnson and Minneman, 1986; Han and Minneman, 1991). The possibility that native  $\alpha_1$ -AR subtypes may form dimers could partially explain such complexity.

Recent studies from our laboratory suggested that epitope-tagged human  $\alpha_1$ -AR subtypes can exist as both monomers and SDS-resistant homodimers and oligomers (Vicentic et al., 2002). In this study, we used biochemical and pharmacological approaches to examine the potential role of dimerization of human  $\alpha_1$ -AR subtypes in controlling their expression and pharmacological properties.

## Materials and Methods

**Materials.** The human  $\alpha_{1A}$ -AR cDNA (Hirasawa et al., 1995) was kindly provided by Dr. Gozoh Tsujimoto (National Children's Hospital, Tokyo, Japan), human  $\alpha_{1B}$ -AR cDNA (Ramarao et al., 1992) was provided by Dr. Dianne Perez (Cleveland Clinic), and human  $\alpha_{1D}$ -AR was cloned in our laboratory (Esbenshade et al., 1995). Other materials were obtained from the following sources: HEK293 cells (American Type Culture Collection, Manassas, VA); fura-2/acetoxymethyl-ester and *n*-dodecyl- $\beta$ -D-maltoside (Calbiochem, La Jolla, CA); (-)-norepinephrine bitartrate, BMY7378, *S*-(+)-niguldipine, Dulbecco's modified Eagle's medium, penicillin, streptomycin, anti-Flag M2 affinity resin, and HRP-conjugated anti-Flag M2 antibody (Sigma Chemical Co., St. Louis, MO); anti-hemagglutinin (HA) affinity matrix (Roche, Indianapolis, IN); anti-HA tag polyclonal antibody and mammalian expression vector pCMV (BD Biosciences Clontech, Palo Alto, CA); sulfo-LC-NHS-biotin, Supersignal enzyme-linked immunosorbent assay Pico chemiluminescent substrate, and Immunopure immobilized streptavidin (Pierce, Rockford, IL); prazosin (Pfizer, Groton, CT); ECL reagent, [ $^{125}$ I]arylazidoprazosin and carrier-free Na [ $^{125}$ I] (Amersham, Chicago, IL). G418 (geneticin), all electrophoresis reagents, and precast 4 to 20% Tris-Glycine polyacrylamide gels were obtained from Invitrogen (Carlsbad, CA), and Superfect Transfection Reagent was from QIAGEN (Valencia, CA).

**Epitope-Tagged  $\alpha_1$ -AR Constructs.** Human  $\alpha_1$ -AR cDNAs were generated by polymerase chain reaction and subcloned into the mammalian expression vector pDT containing in-frame N-terminal hexahistidine and Flag epitope tags as described previously (Robeva et al., 1996; Vicentic et al., 2002). These subtypes were also subcloned into the mammalian expression vector pCMV containing an in-frame N-terminal HA tag (YPYDVPDYA). After sequencing, unique restriction enzyme sites were used to replace the Flag-hexahistidine tag in pDT with the HA-epitope tag to allow generation of stable cell lines under the selection of 400  $\mu$ g/ml G418.

**Cell Culture and Transfection.** HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5%  $CO_2$  (Esbenshade et al., 1993). Cells were transfected with the tagged human  $\alpha_1$ -AR subtypes by calcium phosphate precipitation (30  $\mu$ g/15-cm plate). Transfected cells were propagated for several weeks in the presence of 400  $\mu$ g/ml G418, and subclones were screened by radioligand binding for receptor expression. HA-tagged receptors were transiently transfected into a stable cell line expressing individual Flag-tagged receptors for receptor coexpression studies using 20 to 30  $\mu$ g of cDNA, and cells were harvested 48 to 72 h after transfection.

**Radioligand Binding and  $Ca^{2+}$  Measurements.** For radioligand binding, confluent 15-cm plates were washed with phosphate-buffered saline (PBS; 20 mM  $NaPO_4$ , 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were collected by centrifugation and homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland). Cell membranes were collected by centrifugation at 30,000g for 20 min and resuspended by homogenization in 1× buffer (25 mM HEPES, 150 mM NaCl, pH 7.4, 5 mM EDTA) with a protease inhibitor cocktail (1 mM benzamidine, 3  $\mu$ M pepstatin, 3  $\mu$ M phenylmethylsulfonyl fluoride, 3  $\mu$ M aprotinin, and 3  $\mu$ M leupeptin). Radioligand binding sites were measured by saturation analysis of specific binding of the  $\alpha_1$ -AR antagonist radioligand [ $^{125}$ I]-BE 2254 ( $^{125}$ IBE; 20–800 pM). Nonspecific binding was defined as binding in the presence of 10  $\mu$ M phentolamine. The pharmacological specificity of radioligand binding sites was determined by displacement of  $^{125}$ IBE (50–70 pM) by selected agonists and antagonists, and data were analyzed by nonlinear regression (Theroux et al., 1996). Intracellular  $Ca^{2+}$  mobilization was measured using fura-2 as described previously (Theroux et al., 1996).

**Photoaffinity Labeling.** Photoaffinity labeling was performed on membranes from HEK293 cells expressing Flag- or HA-tagged  $\alpha_1$ -adrenoceptors. Membranes were prepared as described above for radioligand binding (1  $\mu$ g protein/ $\mu$ l), and treated in the dark for 1 h at room temperature with 6 nM [ $^{125}$ I]arylazidoprazosin (Vicentic et al., 2002). Nonspecific labeling was determined in the presence of 1  $\mu$ M unlabeled prazosin. While still in the dark, open tubes were exposed to 6000  $\mu$ J/cm<sup>2</sup> ultraviolet light for 3 min using a Stratalinker. Membranes were then washed once with 1 ml of 1× buffer A with protease inhibitors, centrifuged at 16,000g for 5 min and the pellet homogenized in 0.5 ml of 2× buffer A (50 mM HEPES, 300 mM NaCl, pH 7.4) containing protease inhibitors. Radiolabeled membranes were solubilized with 2% *n*-dodecyl- $\beta$ -D-maltoside (D $\beta$ M) in 1× buffer supplemented with protease inhibitor cocktail for 2 h at 4°C with gentle agitation. After solubilization, the soluble fractions (16,000g) and remaining insoluble material were counted on an automatic gamma counter (1470; PerkinElmer Wallac, Gaithersburg, MD).

**Solubilization and Immunoprecipitation.** Membrane preparations (2–3 mg protein) were prepared as described above and solubilized with 2% D $\beta$ M in 1× buffer supplemented with protease inhibitor cocktail for 2 h at 4°C with gentle agitation. After solubilization, samples were centrifuged at 16,000g or 100,000g and the supernatant was diluted to 0.2% D $\beta$ M in 1× buffer 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, deglycosy-

lated on the resin with *N*-glycosidase F (PNGase F) with gentle agitation for 2 h at room temperature, and then eluted with 4× sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 5% β-mercaptoethanol).

**Western Blots.** Immunoprecipitated samples were run on 4 to 20% Tris-glycine SDS-PAGE, transferred to nitrocellulose, and membranes blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature or overnight at 4°C with gentle agitation. Membranes were then incubated with the appropriate concentration of HRP-conjugated M2-anti-Flag antibody or anti-HA-polyclonal antibody for 1 h at room temperature. Membranes were washed with TBST and detected with ECL or incubated with the appropriate concentration of secondary IgG antibody and then detected with ECL.

**Biotinylation of Cell Surface α<sub>1</sub>-ARs.** Before membrane preparation, intact HEK293 cells expressing single Flag-α<sub>1</sub>-AR subtypes or coexpressing different Flag- and HA-tagged α<sub>1</sub>-ARs were rinsed with PBS and treated with 1.7 mg/ml Sulfo-LC-NHS-biotin, a membrane impermeant biotinylation agent, for 30 min at room temperature. The reaction was quenched by incubating cells with 500 mM Tris-HCl, pH 7.5, for 5 min at room temperature. Membranes were prepared and solubilized as described above and precipitated with immobilized streptavidin or anti-Flag affinity resin overnight at 4°C with gentle agitation. Resin was collected by centrifugation, washed with 1× buffer, receptors were deglycosylated with PNGase F on the resin with gentle agitation for 2 h at room temperature, and eluted with 4× sample buffer. Fractions (30 μl) were separated by SDS-PAGE, transferred to nitrocellulose, and blocked as described above. Membranes were incubated with HRP-conjugated M2-anti-Flag antibody or HRP-conjugated streptavidin, washed with TBST, and detected with ECL.

**Surface Expression Assay.** Stably transfected HEK293 cells were transiently transfected with tagged human α<sub>1</sub>-AR subtypes by Superfect transfection reagent (10 μg/10-cm plate). One day after transfection, cells were split into poly-D-lysine-coated 35-mm dishes (Biocoat) and grown overnight at 37°C. The cells were rinsed in PBS, fixed with 4% paraformaldehyde in PBS for 30 min, and then rinsed three times in PBS. The cells were blocked with blocking buffer (2% nonfat milk in PBS, pH 7.4) for 30 min and then incubated with the appropriate concentration of HRP-conjugated M2-anti-Flag antibody in blocking buffer for 1 h at room temperature. The cells were washed three times with blocking buffer, then once with PBS, and then incubated with enzyme-linked immunosorbent assay ECL reagent for 15 s. The luminescence, which corresponds to the amount of receptor on the cell surface (Xu et al., 2003), was determined by placing the plate inside a TD20/20 luminometer (Turner Designs, Sunnyvale, CA).

## Results

**Characterization of HA-Tagged α<sub>1</sub>-AR Subtypes.** We made N-terminal HA-tagged human α<sub>1</sub>-AR subtypes for use with our previously characterized Flag-tagged subtypes (Vicentic et al., 2002) to directly examine homo- and het-

erodimerization. Constructs were expressed in HEK293 cells, which do not endogenously express detectable α<sub>1</sub>-ARs, and the density of recombinant receptor expression was determined by radioligand binding to be 0.3 to 16 pmol/mg of protein (Table 1). The three HA-α<sub>1</sub>-AR subtypes exhibited similar *K<sub>D</sub>* values for the radioligand <sup>125</sup>IBE (Table 1), which were similar to *K<sub>D</sub>* values reported for <sup>125</sup>IBE at both wild-type and Flag-tagged human α<sub>1</sub>-ARs (Esbenshade et al., 1993; Han and Minneman, 1991; Vicentic et al., 2002). The pharmacological specificities of the binding sites were also unaffected by the N-terminal tag, because *K<sub>I</sub>* values for the agonist NE, the nonsubtype selective antagonist prazosin, the α<sub>1A</sub>-selective antagonist (+)-niguldipine, and the α<sub>1D</sub>-selective antagonist BMY7378 in competing for specific <sup>125</sup>IBE binding to HA-tagged subtypes showed no significant differences from those observed at wild-type and Flag-tagged α<sub>1</sub>-AR subtypes (Table 1) (Han and Minneman, 1991; Esbenschade et al., 1993; Lei et al., 2002). HA-tagged subtypes also showed NE-stimulated increases in intracellular Ca<sup>2+</sup> (data not shown). Epitope-tagged receptor proteins were examined by Western blots after solubilization, immunoprecipitation, and deglycosylation (Fig. 1). An anti-HA antibody detected what seemed to be both monomers (α<sub>1A</sub>, ~50 kDa; α<sub>1B</sub>, ~65 kDa; α<sub>1D</sub>, ~80 kDa) and dimers (~100,130,160 kDa) and oligomers (~150–240 kDa) of each receptor as observed previously with Flag-α<sub>1</sub>-AR subtypes (Vicentic et al., 2002).

**Homodimerization of α<sub>1</sub>-AR Subtypes.** To obtain direct evidence for α<sub>1</sub>-AR homodimerization, each HA-tagged α<sub>1</sub>-AR subtype was transiently transfected into HEK293 cells (100–500 fmol/mg) stably expressing the same subtype containing a Flag tag (either Flag-α<sub>1A</sub>, Flag-α<sub>1B</sub>, or Flag-α<sub>1D</sub>; 150–600 fmol/mg). Membranes from cotransfected cells were solubilized and immunoprecipitated with anti-Flag or anti-HA antibody. After elution and separation on gels, proteins were visualized by Western blots with anti-Flag antibody or anti-HA antibody. Figure 2 shows that the anti-Flag antibody apparently detected monomers (α<sub>1A</sub>, ~50 kDa; α<sub>1B</sub>, ~65 kDa; α<sub>1D</sub>, ~80 kDa), dimers and oligomers (~110–240 kDa) of all three subtypes in immunoprecipitates from either anti-Flag or anti-HA antibody (Fig. 2). Because no cross-reactivity between the two antibodies and tags was observed (data not shown), Flag-α<sub>1</sub>-ARs must have coprecipitated with HA-α<sub>1</sub>-ARs, supporting the existence of receptor homodimers and oligomers. Similar results were obtained when coexpressed receptors were detected with anti-HA antibody (data not shown).

**Subtype-Specific Heterodimerization of α<sub>1</sub>-AR Subtypes.** Differentially tagged combinations of α<sub>1</sub>-AR subtypes were coexpressed at similar levels in HEK293 cells, and

TABLE 1

Binding properties of HA-tagged α<sub>1</sub>-AR subtypes expressed in HEK293 cells

α<sub>1</sub>-AR subtypes with N-terminal HA tags were stably expressed in HEK293 cells, membranes were harvested, and saturation of specific <sup>125</sup>IBE binding measured as described under *Materials and Methods*. *B<sub>max</sub>* and *K<sub>D</sub>* values were calculated by nonlinear regression of saturation curves, and *pK<sub>I</sub>* values for inhibition of binding by NE, prazosin, (+)-niguldipine, and BMY7378 were determined. Values are presented as mean ± S.E.M. of three experiments performed in duplicate.

Subtypes expressed	<i>B<sub>max</sub></i> <i>fmol/mg</i>	<i>K<sub>D</sub></i> <i>pM</i>	<i>pK<sub>I</sub></i>			
			NE	Prazosin	(+)-Niguldipine	BMY7378
HA-α <sub>1A</sub>	1364 ± 193	45 ± 12	4.9 ± 0.12	9.0 ± 0.15	9.3 ± 0.04	6.0 ± 0.08
HA-α <sub>1B</sub>	16165 ± 1050	38 ± 13	5.0 ± 0.09	9.3 ± 0.09	6.9 ± 0.04	6.1 ± 0.09
HA-α <sub>1D</sub>	331 ± 10	59 ± 14	5.5 ± 0.14	9.4 ± 0.27	6.9 ± 0.35	8.3 ± 0.19



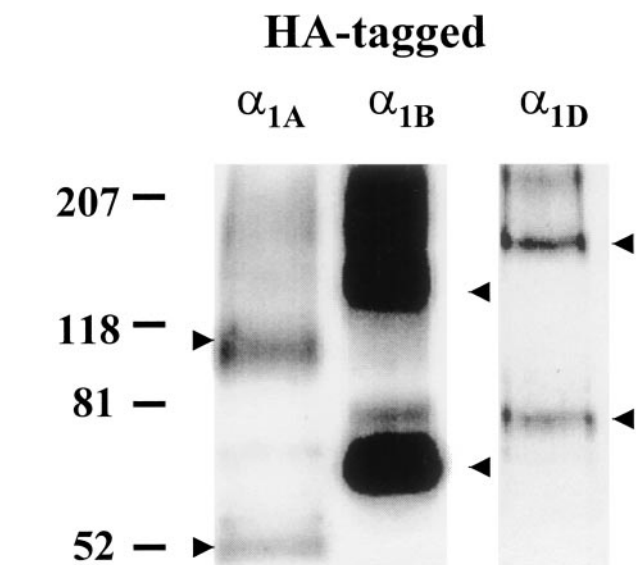
coimmunoprecipitation was used to determine whether these subtypes form heterodimers. In Fig. 3, left, HA- $\alpha_{1B}$ -ARs were transiently transfected into HEK293 cells (~300–450 fmol/mg) stably expressing Flag- $\alpha_{1A}$ - or Flag- $\alpha_{1D}$ -ARs (150–600 fmol/mg). Expression levels of each subtype were within physiological levels (Schwinn, 1994; Graham et al., 1996; Michelotti et al., 2000). Membranes from these cotransfected

cells were solubilized, immunoprecipitated with anti-Flag or anti-HA antibodies, and Western blots performed with anti-Flag or anti-HA antibodies. Figure 3 demonstrates an interaction between coexpressed Flag- $\alpha_{1A}$  and HA- $\alpha_{1B}$ -ARs. As expected after anti-Flag immunoprecipitation, the anti-Flag antibody detected bands migrating as monomers (52 kDa) and dimers (110 kDa) of Flag- $\alpha_{1A}$ . After anti-HA immunoprecipitation, anti-Flag antibody also detected monomers and dimers of Flag- $\alpha_{1A}$ , presumably caused by association with HA- $\alpha_{1B}$  (Fig. 3, Left). After anti-HA immunoprecipitation, the HA- $\alpha_{1B}$  seemed as both monomers and dimers of a higher molecular mass, as expected.

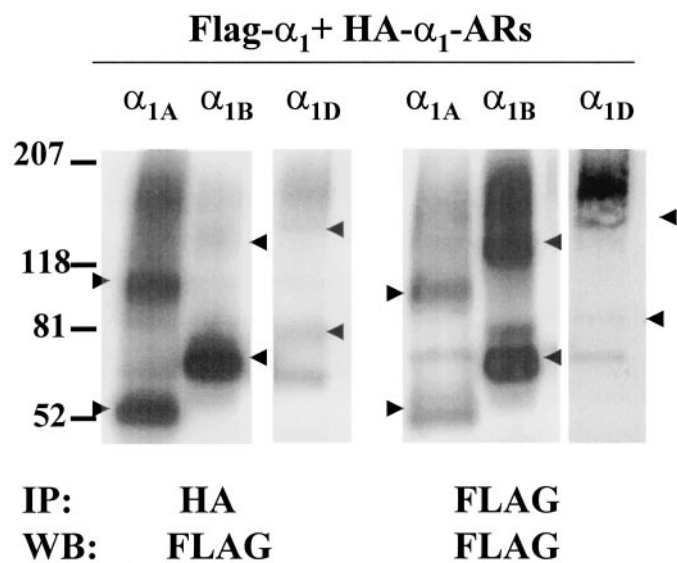
Figure 3, center, also shows interactions between coexpressed Flag- $\alpha_{1D}$  and HA- $\alpha_{1B}$ -ARs. After coexpression, solubilization, and immunoprecipitation with anti-Flag antibody, monomers (80 kDa), dimers (160 kDa), and trimers (240 kDa) of Flag- $\alpha_{1D}$ -ARs were apparent as observed previously. After anti-HA immunoprecipitation, anti-Flag antibody also detected similar Flag- $\alpha_{1D}$ -ARs, indicating interactions between Flag- $\alpha_{1D}$  and HA- $\alpha_{1B}$ -ARs (Fig. 3, center). Dimers and oligomers were also observed after anti-HA precipitation. As expected, after immunoprecipitation with anti-HA antibody the HA- $\alpha_{1B}$  seemed as both monomers and dimers on an anti-HA blot, which were smaller than Flag- $\alpha_{1D}$ -ARs.

Because  $\alpha_{1B}$ -ARs formed heterodimers with both  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs, we wanted to determine whether  $\alpha_{1A}/\alpha_{1D}$ -AR heterodimers could also be detected. HA- $\alpha_{1A}$ -ARs were transiently expressed (~200–300 fmol/mg) into stably expressing Flag- $\alpha_{1D}$ -AR HEK293 cells (~150–300 fmol/mg), solubilized, and immunoprecipitated with anti-HA or anti-Flag antibodies. Figure 3 (right) shows that after immunoprecipitation with anti-HA antibody, Flag- $\alpha_{1D}$ -ARs were not detected by anti-Flag antibody, even after long exposure times. However, parallel blots of anti-Flag immunoprecipitated Flag- $\alpha_{1D}$ -ARs and anti-HA immunoprecipitated HA- $\alpha_{1A}$ -ARs detected bands consistent with monomers and oligomers of each receptor, respectively (Fig. 3, right), confirming that these receptors were efficiently expressed at similar levels. Taken together, these results suggest that interactions between  $\alpha_1$ -ARs are restricted to particular combinations of subtypes.

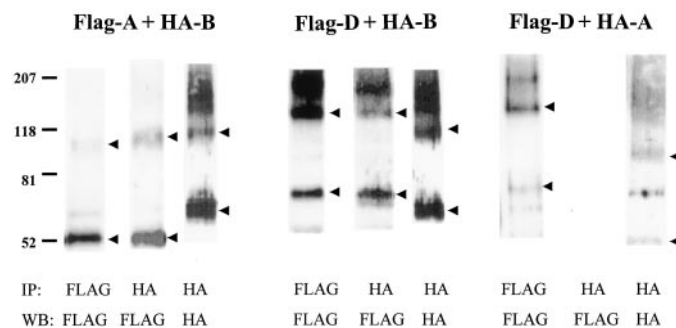
To determine whether these observations were caused by



**Fig. 1.** Western blot analysis of HA-tagged- $\alpha_1$ -ARs expressed in HEK293 cells. Membranes from cells stably expressing each HA-tagged subtype were solubilized and immunoprecipitated with anti-HA affinity resin. Samples were deglycosylated with PNGase F, subjected to SDS-PAGE, transferred to nitrocellulose, blotted with anti-HA polyclonal antibody, and detected with ECL. Arrows indicate apparent monomers and dimers of each subtype, and the figure is representative of three or four experiments.



**Fig. 2.** Coimmunoprecipitation of HA- $\alpha_1$ -AR subtypes transiently coexpressed in HEK293 cells stably expressing the same Flag- $\alpha_1$ -AR subtype. Cells coexpressing HA- and Flag-tagged  $\alpha_{1A}$ -; HA- and Flag-tagged  $\alpha_{1B}$ -; or HA- and Flag- $\alpha_{1D}$ -ARs were harvested and membrane preparations solubilized and immunoprecipitated (IP) with anti-HA (left) or anti-Flag (right) affinity resin as indicated. Samples were deglycosylated, and Western blots (WB) performed with HRP-anti-Flag antibody. Arrows indicate apparent monomers and dimers of each subtype, and each blot is representative of three or four experiments.



**Fig. 3.** Immunoprecipitation of HA-tagged  $\alpha_1$ -AR subtypes transiently coexpressed with stable Flag-tagged  $\alpha_1$ -AR subtypes. Left, Flag- $\alpha_{1A}$ - and HA- $\alpha_{1B}$ -ARs coexpressed in HEK293 cells. Center, coexpressed Flag- $\alpha_{1D}$ -ARs and HA- $\alpha_{1B}$ -ARs. Right, coexpressed Flag- $\alpha_{1D}$ -ARs and HA- $\alpha_{1A}$ -ARs. Membrane preparations from cells expressing different combinations of subtypes were solubilized and immunoprecipitated (IP) with anti-Flag or anti-HA affinity resin as indicated. Samples were deglycosylated, electrophoresed and transferred, and Western blots (WB) were performed with anti-Flag or anti-HA antibodies. Arrows indicate apparent monomers and dimers of each receptor. This blot is representative of three or four experiments.

interactions of these highly hydrophobic proteins occurring after solubilization, different HEK293 cell lines, each expressing only one (Flag- $\alpha_{1A}$  or HA- $\alpha_{1B}$ ) tagged subtype, were solubilized, mixed together, and sonicated again to allow the two receptor populations to come together in the same micelle. When these mixtures were immunoprecipitated with anti-HA antibody, anti-Flag antibody did not detect Flag- $\alpha_{1A}$ -AR on Western blots (Fig. 4A, left); and when the mixture was immunoprecipitated with anti-Flag antibody, anti-HA antibody did not detect HA- $\alpha_{1B}$ -ARs (Fig. 4A, right). Similar results were seen with Flag- $\alpha_{1D}$ - and HA- $\alpha_{1B}$ -AR mixtures (not shown), demonstrating that these interactions are not solubilization artifacts, and that receptors must be coexpressed in cells to interact.

**Extent of Solubilization of Epitope-Tagged  $\alpha_1$ -ARs.** Membranes from HEK293 cells stably expressing epitope-tagged  $\alpha_1$ -ARs were solubilized with 2% D $\beta$ M, which has previously been used to effectively solubilize G-protein coupled receptors (Gether et al., 1995). We used photoaffinity

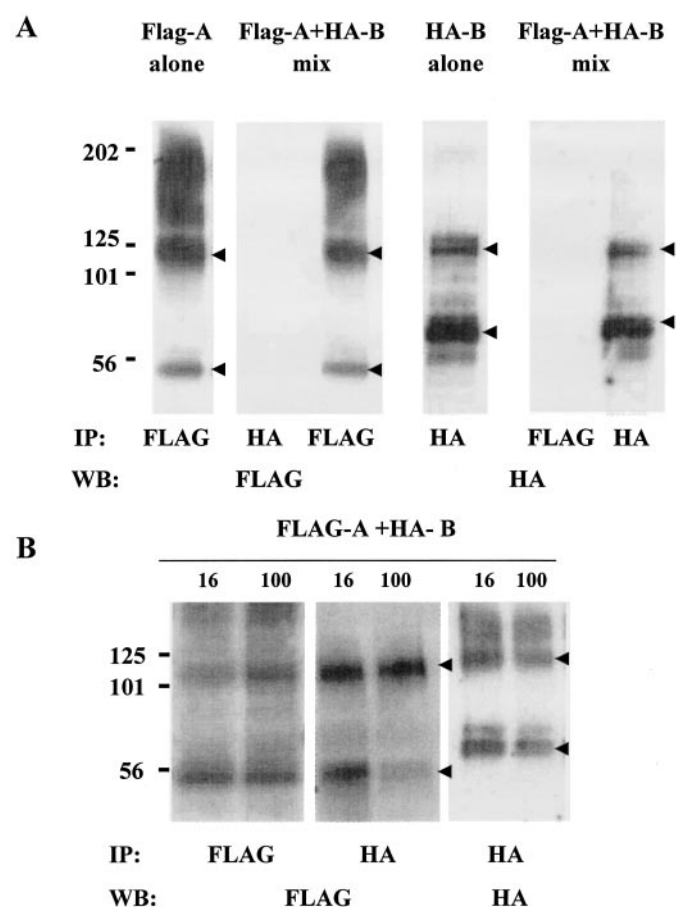
labeling to determine the efficiency of D $\beta$ M solubilization of  $\alpha_1$ -ARs. Receptors were covalently labeled with [ $^{125}$ I]arylazidoprazosin before solubilization, and the proportion of labeled receptor solubilization was determined. Membranes expressing  $\alpha_{1A}$ -ARs,  $\alpha_{1B}$ -ARs, or a combination of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs were labeled with [ $^{125}$ I]arylazidoprazosin, and total radioactivity incorporated was determined before and after solubilization and centrifugation. We found that 83, 86, and 88% of  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1A} + \alpha_{1B}$ -ARs, respectively, were found in the soluble fraction, showing that a large majority of photoaffinity-labeled receptors are solubilized with this protocol. Because of the low degree of photoaffinity labeling of  $\alpha_{1D}$ -ARs (Vicentic et al., 2002), the degree of solubilization of this subtype was not determined.

Ultracentrifugation was also used to determine whether these receptors might be only partially solubilized. Solubilized samples were ultracentrifuged at 100,000g and compared by Western blot analysis to soluble fractions spun at our standard 16,000g. Figure 4B shows that there are no differences between solubilized Flag- $\alpha_{1A}$ - and HA- $\alpha_{1B}$ -ARs after 100,000g or 16,000g spins when immunoprecipitated with anti-Flag or anti-HA resin and blotted with anti-Flag or anti-HA antibodies. Similar results were seen with the other  $\alpha_1$ -AR combinations (not shown), demonstrating that these interactions are not caused by partial receptor solubilization.

**Effect of Agonist Activation on Dimerization.** To determine whether agonist occupancy altered homodimer formation, HEK293 cells expressing single Flag- $\alpha_1$ -AR subtypes were pretreated with 100  $\mu$ M NE for 15 min at 37°C. Western blots of immunoprecipitated samples from both stimulated and nonstimulated cells were quantitated by densitometry and relative band intensities determined. These experiments showed that NE did not significantly alter either the density of the bands on Western blots (not shown) or the ratios of monomers to higher order oligomers (Fig. 5A).

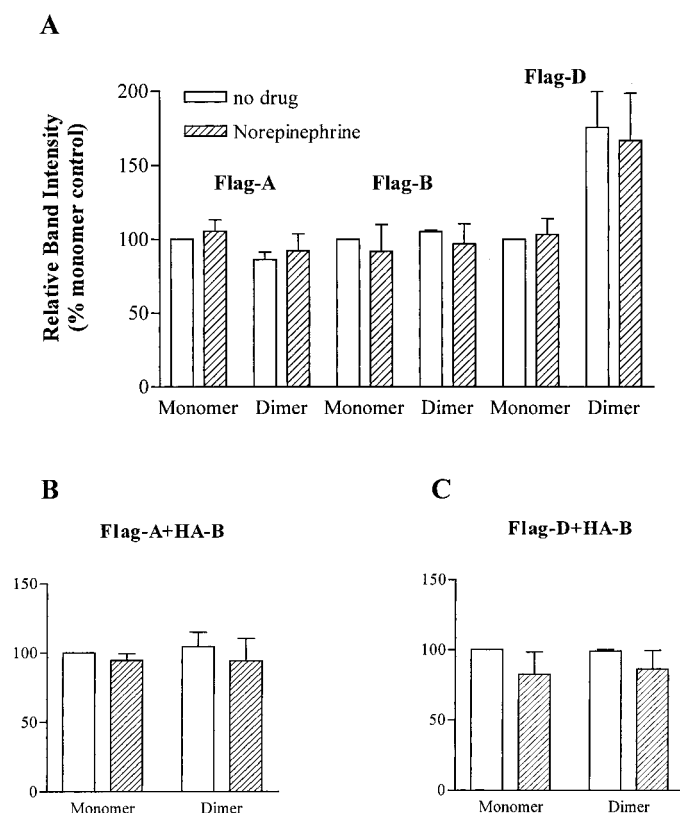
Similar experiments were performed to determine whether agonist occupancy altered heterodimer formation. HEK293 cells coexpressing HA- $\alpha_{1B}$ - (~300–450 fmol/mg) and Flag- $\alpha_{1A}$ - or Flag- $\alpha_{1D}$ -ARs (150–600 fmol/mg) were pretreated with 100  $\mu$ M NE for 15 min at 37°C before lysis. Solubilized fractions were immunoprecipitated with anti-HA affinity resin, and Western blots were performed with anti-Flag antibody. Blots were quantitated by densitometry, showing that NE did not alter either expression (not shown), or the extent of heterodimer formation for the Flag-tagged subtype in either combination (Fig. 5, B and C). NE also did not induce heterodimer formation between  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs (data not shown). These results indicate that dimerization of  $\alpha_1$ -AR subtypes is independent of agonist activation.

**Effect of C-Terminal Truncation on Dimerization.** To examine a possible role for the intracellular C terminus in  $\alpha_1$ -AR dimerization, we constructed C-terminal truncation mutants of each Flag- $\alpha_1$ -AR subtype. Truncations were made 14 to 16 amino acids (aa) after the end of the predicted 7th transmembrane domain, at a conserved glutamine ( $\alpha_{1A}$ , Gln344;  $\alpha_{1B}$ , Gln366) or adjacent arginine ( $\alpha_{1D}$ , Arg418) by mutation into a stop codon, and C-truncated (Ctr) Flag- $\alpha_1$ -ARs stably expressed in HEK293 cells. In Fig. 6A, it is apparent that after solubilization and immunoprecipitation with anti-Flag antibody, Western blots with anti-Flag antibody detected bands migrating as monomers and dimers of the Flag-Ctr $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -ARs. As expected, C-trun-



**Fig. 4.** A, lack of interaction between presolubilized  $\alpha_1$ -AR subtypes. Membrane preparations from HEK293 cells expressing only Flag- $\alpha_{1A}$  or only HA- $\alpha_{1B}$ -ARs were solubilized, mixed together, re-sonicated, and immunoprecipitated (IP) with anti-Flag or anti-HA affinity resin as indicated. Samples were deglycosylated with PNGase F, and Western blots (WB) were performed with anti-Flag antibody (left) or anti-HA antibody (right). B, membrane preparations from HEK293 cells coexpressing Flag- $\alpha_{1A}$  and HA- $\alpha_{1B}$ -ARs were solubilized, centrifuged at 16,000g (16) or 100,000g (100) and immunoprecipitated (IP) with anti-Flag or anti-HA affinity resin as indicated. Samples were deglycosylated, and Western blots (WB) were performed with anti-Flag or anti-HA antibody as indicated. Arrows indicate apparent monomers and dimers of each receptor. Results are representative of three or four experiments.

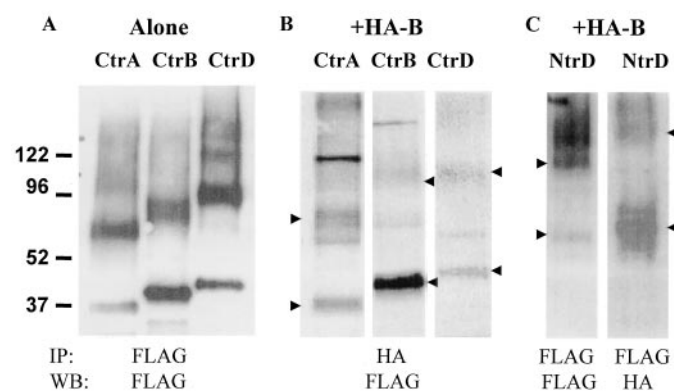
cated mutants were about 30% smaller than full-length receptors, with the corresponding monomers running around 36, 40, and 45 kDa and dimers around 75 to 100 kDa. Full-length HA- $\alpha_{1B}$ -ARs (~300–450 fmol/mg) were then transfected into HEK293 cells stably expressing Flag-Ctr- $\alpha_{1A}$ , Flag-Ctr- $\alpha_{1B}$ , or Flag-Ctr- $\alpha_{1D}$ -ARs at physiological levels (100–400 fmol/mg). After solubilization and immunoprecipitation with anti-HA affinity resin and deglycosylation, Western blots were performed with anti-Flag antibody. Figure 6B shows that anti-Flag antibody detected monomers and dimers of all three C-truncated mutants after immunoprecipitation with anti-HA antibody, indicating interactions between full-length HA- $\alpha_{1B}$ -ARs and each Flag-Ctr- $\alpha_1$ -AR subtype. The size of the anti-HA coprecipitated Ctr- $\alpha_1$ -ARs were similar to those observed after anti-Flag immunoprecipitation, although the ratio of monomers to oligomers was higher in the anti-HA versus anti-Flag immunoprecipitated samples. These results suggest that full-length  $\alpha_{1B}$ -ARs form dimers with  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, or  $\alpha_{1D}$ -AR C-truncated mutants, supporting the hypothesis that the C-terminal domain is not critical for this interaction.



**Fig. 5.** Lack of effect of NE on dimerization of  $\alpha_1$ -AR subtypes. Intact cells expressing one or more tagged subtypes were serum starved for 2 h and treated with or without 100  $\mu$ M NE for 15 min. Membranes were harvested, solubilized, immunoprecipitated, and blotted with anti-Flag antibody. Western blots (not shown) were analyzed by densitometry for monomers and dimers as determined by their apparent molecular masses. A, percentage relative band intensity comparing distribution of stimulated and unstimulated Flag- $\alpha_{1A}$ -, Flag- $\alpha_{1B}$ -, or Flag- $\alpha_{1D}$ -ARs expressed in isolation. B and C, Flag- $\alpha_{1A}$ -ARs or Flag- $\alpha_{1D}$ -ARs were co-transfected with HA- $\alpha_{1B}$ -ARs, immunoprecipitated with anti-HA affinity resin, and blotted with anti-Flag antibody. The percentage of relative band intensities of the Flag-tagged subtypes in the presence or absence of NE were quantitated. The density of each band is expressed as a percentage of the density of the unstimulated monomer band (set as 100%). Data represents mean  $\pm$  S.E.M. of three independent experiments.

**Effect of N-Terminal Truncation on  $\alpha_{1D}$ -AR Dimerization.**  $\alpha_{1D}$ -ARs have an unusually long extracellular N terminus (95 aas) compared with  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs (25 and 45 aa). We examined the possibility that the N terminus of  $\alpha_{1D}$ -ARs might be involved in heterodimerization by generating mutants truncated at Gly<sup>79</sup> (Pupo et al., 2003). N-Truncated- $\alpha_{1D}$  coding sequence was subcloned into the expression vector pDT containing an N-terminal Flag tag, and Flag-Ntr- $\alpha_{1D}$ -ARs were transiently coexpressed with full-length HA- $\alpha_{1B}$ -ARs in HEK293 cells. After immunoprecipitation with anti-Flag antibody, Western blots were performed with anti-Flag or anti-HA antibodies. Figure 6C shows that anti-Flag antibody detected monomers and dimers of Flag-Ntr- $\alpha_{1D}$ -ARs with approximate molecular masses of 55 and 110 kDa, as previously reported (Pupo et al., 2003). Figure 6C also shows that anti-HA antibody detected monomers and dimers of full-length HA- $\alpha_{1B}$ -ARs immunoprecipitated by the anti-Flag antibody, indicating an interaction between the full-length HA- $\alpha_{1B}$  and Flag-Ntr- $\alpha_{1D}$ -ARs, suggesting that the N terminus is not required for heterodimerization.

**Biotinylation of Monomers and Higher Order Oligomers.** To determine whether  $\alpha_1$ -AR homodimers were present on the cell surface, intact HEK293 cells expressing single Flag- $\alpha_1$ -AR subtypes were treated with the membrane-impermeant biotinylating reagent sulfo-LC-NHS-biotin before membrane preparation. After solubilization, biotinylated proteins were precipitated with streptavidin, deglycosylated, and eluted with 4 $\times$  sample buffer. Western blots of streptavidin-purified samples were performed with anti-Flag antibody to determine which receptor species were subject to cell-surface biotinylation. Monomers of all three receptors corresponding to their predicted molecular masses ( $\alpha_{1A}$ , ~50 kDa;  $\alpha_{1B}$ , ~65 kDa;  $\alpha_{1D}$ , ~80 kDa) could be detected in the surface biotinylated samples (Fig. 7, top left), although the  $\alpha_{1A}$  signal was weak and required a long exposure time for detection. In addition, homodimers and trimers of all three subtypes, corresponding to their predicted molecular masses, were detected in the streptavidin-purified samples. To compare with total receptor expression, parallel samples



**Fig. 6.** Western blot analysis of Flag- $\alpha_1$ -AR C-terminal (Ctr) or N-terminal (Ntr) truncation mutants. Flag-tagged Ctr- $\alpha_{1A}$ , Ctr- $\alpha_{1B}$ , or Ctr- $\alpha_{1D}$ -ARs (CtrA, CtrB, CtrD) were expressed alone (A) or coexpressed (B) with full-length HA- $\alpha_{1B}$ -ARs (HA-B) in HEK293 cells. C, Flag-tagged N-truncated  $\alpha_{1D}$ -ARs (NtrD) were coexpressed with full-length HA- $\alpha_{1B}$ -ARs (HA-B) in HEK293 cells. Membrane preparations were solubilized and immunoprecipitated (IP) with anti-Flag or anti-HA affinity resin as indicated. Samples were deglycosylated and Western blots (WB) performed with HRP-anti-Flag or anti-HA antibody. Arrows in B and C indicate apparent monomers and dimers of each subtype. Results are representative of three or four experiments.



were immunoprecipitated with anti-Flag affinity resin, and blotted with anti-Flag antibody. All three anti-Flag immunoprecipitated receptors were also visible as monomers, dimers and trimers (Fig. 7, top right). Sequential coimmunoprecipitations were also done to further determine which receptor complexes were present on the cell surface. In these experiments, biotinylated  $\alpha_1$ -AR samples were immunoprecipitated with anti-Flag and then anti-HA resin before detection with streptavidin on Western blots. However, no bands were detected on these blots (data not shown) even after long exposure times, probably because of poor recovery of samples after this complex procedure.

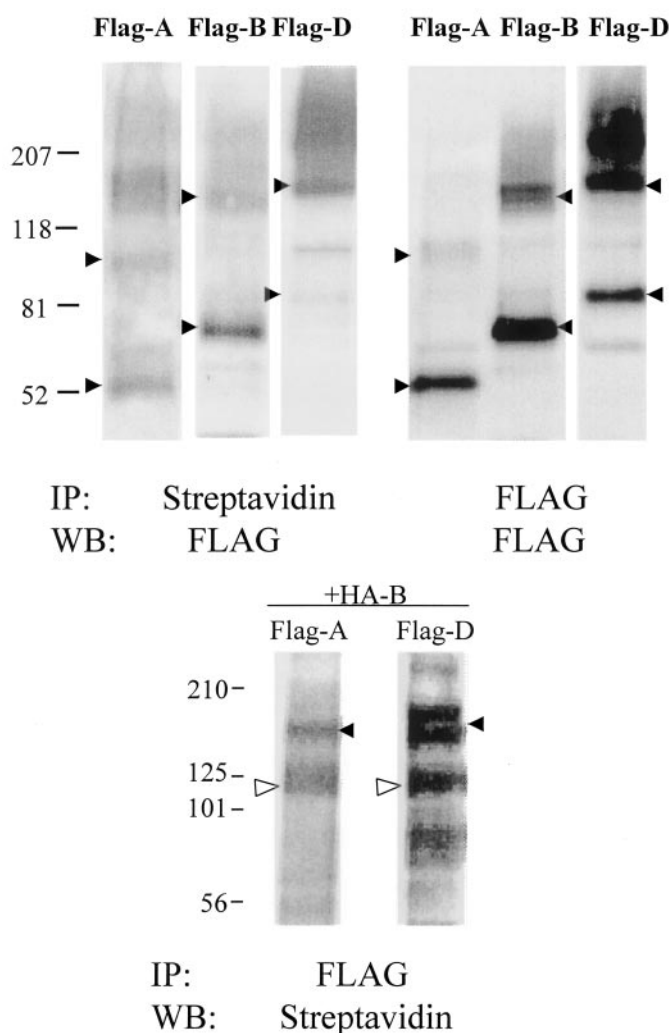
To determine whether heterodimers were present on the

cell surface, cells cotransfected with Flag- $\alpha_{1A}$ - and HA- $\alpha_{1B}$ -ARs, or cotransfected with Flag- $\alpha_{1D}$ - and HA- $\alpha_{1B}$ -ARs, were also treated with sulfo-LC-NHS-biotin. After solubilization, biotinylated proteins were immunoprecipitated with anti-Flag affinity resin, deglycosylated, eluted with sample buffer, and blotted with HRP-streptavidin. In Fig. 7, bottom, high molecular mass species apparently corresponding to trimeric complexes of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs ( $\sim 150$ – $160$  kDa) were detected by streptavidin on the cell surface, along with apparent HA- $\alpha_{1B}$ -AR dimers ( $\sim 125$  kDa). After a long exposure, dimers of Flag- $\alpha_{1A}$ -ARs were also detected on the cell surface (data not shown). Figure 7, bottom, also shows large molecular mass species consistent with heterodimers and -trimers of  $\alpha_{1B}$ - and  $\alpha_{1D}$ -ARs ( $\sim 145$ – $200$  kDa) detected by streptavidin, along with apparent HA- $\alpha_{1B}$ -AR homodimers. These data indicate that oligomeric complexes of  $\alpha_{1B}/\alpha_{1A}$ -ARs and  $\alpha_{1B}/\alpha_{1D}$ -ARs are expressed on the cell surface, although it is difficult to determine, in these higher order complexes, which receptors are actually present and in what proportion.

**Pharmacological Characterization of  $\alpha_1$ -AR Heterodimers.** The binding properties of heterodimers were determined using the antagonist radioligand  $^{125}$ IBE. The density of binding sites ( $B_{\max}$ ) and affinity ( $K_D$ ) for  $^{125}$ IBE were determined by saturation analysis of specific  $^{125}$ IBE binding in membranes from cells transfected with different combinations of tagged subtypes expressed alone or together (Tables 2 and 3), with each experiment representing three to four different transfections. No significant differences in affinity for  $^{125}$ IBE were found with any combinations of subtypes examined (Tables 2 and 3).

Inhibition of specific  $^{125}$ IBE binding by the agonist NE, the non-subtype-selective antagonist prazosin, the  $\alpha_{1A}$ -selective antagonist (+)-niguldipine, or the  $\alpha_{1D}$ -selective antagonist BMY7378 was examined in each preparation, and  $K_I$  values were determined by nonlinear regression analysis (Fig. 8, Table 4). We found no changes in apparent  $K_D$  values for prazosin, which binds to all cloned subtypes with similar affinities, when combinations of  $\alpha_1$ -AR subtypes were coexpressed. However, the subtype-selective antagonists (+)-niguldipine and BMY7378 showed different affinities for individual subtypes, as previously reported, and competition curves for these drugs in cells expressing combinations of subtypes were clearly biphasic (Fig. 8). Biphasic curves were separated into two components by nonlinear regression analysis to determine the fraction of high- and low-affinity binding sites and the affinity of the competing drug for each site.

When  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs were coexpressed, two-site analysis of inhibition of specific  $^{125}$ IBE binding by (+)-niguldipine (Fig. 8A) showed that 68% of binding sites had a high affinity characteristic of  $\alpha_{1A}$ -ARs. Receptor density in HEK293 cells expressing only  $\alpha_{1A}$ -ARs, only  $\alpha_{1B}$ -ARs, or a combination of  $\alpha_{1A} + \alpha_{1B}$ -ARs is shown in Table 2, and the density of each subtype was calculated by multiplying total  $B_{\max}$  by the fraction of sites showing high or low affinity for (+)-niguldipine.  $\alpha_{1A}$ - or  $\alpha_{1B}$ -ARs expressed alone showed similar  $B_{\max}$  values (Table 2), however the density of  $\alpha_{1A}$ -ARs was significantly increased (by approximately 66%) when coexpressed with  $\alpha_{1B}$ -ARs, whereas  $\alpha_{1B}$ -AR density was not significantly altered (Table 2). Densitometric analysis of Western blots of samples immunoprecipitated with anti-Flag antibody also showed an  $83 \pm 1.9\%$  increase in signal strength for Flag-



**Fig. 7.** Surface expression of Flag- $\alpha_1$ -AR subtypes detected with a membrane-impermeant biotinylating reagent. Intact HEK293 cells expressing individual Flag- $\alpha_1$ -AR subtypes (top) or a combination of subtypes as indicated (bottom) were treated with sulfo-LC-NHS-biotin and harvested, and membranes were prepared. Membranes were solubilized and precipitated (IP) with either immobilized streptavidin or anti-Flag affinity resin, as indicated. Samples were deglycosylated and eluted with 4 $\times$  sample buffer. Equal amounts of protein (in 30  $\mu$ l) were loaded onto Western blots (WB) detected with HRP-anti-Flag antibody (top) or HRP-streptavidin (bottom). Closed arrows indicate apparent monomers and dimers of each receptor (top) or apparent heterooligomers of Flag- $\alpha_{1A}$ - + HA- $\alpha_{1B}$ -ARs or Flag- $\alpha_{1D}$ - + HA- $\alpha_{1B}$ -ARs (bottom); hollow arrow indicates apparent HA- $\alpha_{1B}$ -AR dimers (bottom) that were observed in both coexpression samples. These blots are representative of two to six experiments.

When  $\alpha_{1D}$ -ARs were coexpressed with either  $\alpha_{1A}$ - or  $\alpha_{1B}$ -ARs, the fraction of binding sites with a high affinity for the  $\alpha_{1D}$ -selective antagonist BMY7378 was used to determine the proportion of  $\alpha_{1D}$ -ARs. Receptor density was determined

TABLE 2

Differentially tagged  $\alpha_{1A}$ -AR subtypes were expressed alone or HA- $\alpha_{1B}$ -ARs were transiently expressed in HEK293 cells stably expressing Flag- $\alpha_{1A}$ -ARs, membranes were harvested, and  $B_{\max}$  and  $K_D$  values were calculated by nonlinear regression of saturation curves of specific [ $^{125}$ I]BE binding. Estimated  $B_{\max}$  values for each coexpressed subtype were calculated from the fraction of high- and low-affinity binding sites for (+)-niguldipine (see Fig. 8). Values are presented as mean  $\pm$  S.E.M. of three independent transfection experiments performed in duplicate.

Subtypes Expressed	$K_D$	$B_{\max}$	Estimated $B_{\max}$	
			Flag- $\alpha_{1A}$	HA- $\alpha_{1B}$
	$pM$	$fmol/mg$	$fmol/mg$	
Flag- $\alpha_{1A}$	$41 \pm 5$	$661 \pm 102$		
HA- $\alpha_{1B}$	$39 \pm 13$	$471 \pm 61$		
Flag- $\alpha_{1A}$ + HA- $\alpha_{1B}$	$45 \pm 12$	$1635 \pm 69$	$1098 \pm 79^a(+66\%^b)$	$537 \pm 15(+14\%^b)$

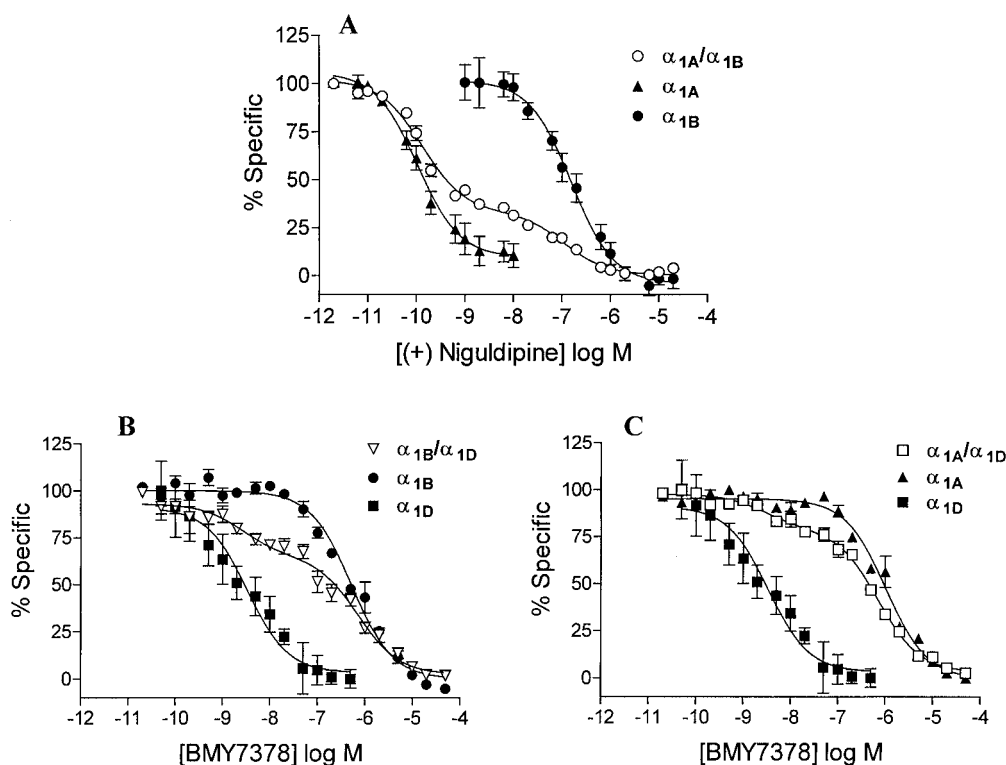
<sup>b</sup> Increase in  $B_{\max}$  compared with the same subtype expressed alone.

TABLE 3

Differentially tagged  $\alpha$ -AR subtypes were expressed alone or HA- $\alpha$ -ARs were transiently expressed in HEK293 cells stably expressing Flag- $\alpha$ -ARs, membranes were harvested, and  $B_{\max}$  and  $K_D$  values calculated by nonlinear regression of saturation curves of specific [ $^{125}$ I]BE binding. Estimated  $B_{\max}$  values for each coexpressed subtype were calculated from the fraction of high- and low-affinity binding sites for BMY7378 (see Fig. 8). Values are presented as mean  $\pm$  S.E.M. of three independent transfection experiments performed in duplicate.

Subtypes Expressed	$K_D$	$B_{\max}$	Estimated $B_{\max}$	
			HA- $\alpha_{1A}$	Flag- $\alpha_{1D}$
	$pM$	$fmol/mg$		$fmol/mg$
HA- $\alpha_{1A}$	$45 \pm 22$	$452 \pm 70$		
HA- $\alpha_{1B}$	$37 \pm 3$	$481 \pm 44$		
Flag- $\alpha_{1D}$	$64 \pm 16$	$273 \pm 7$		
Flag- $\alpha_{1D}$ + HA- $\alpha_{1B}$	$50 \pm 16$	$1513 \pm 121$		$1052 \pm 84^a (+119\%^b)$
Flag- $\alpha_{1D}$ + HA- $\alpha_{1A}$	$49 \pm 7$	$644 \pm 107$	$532 \pm 92 (+14\%^b)$	$458 \pm 44^a (+68\%^b)$ $179 \pm 25 (-34\%^b)$

<sup>b</sup> Increase in  $B_{\max}$  compared with the same subtype expressed alone.



**Fig. 8.** Inhibition of specific  $^{125}\text{I}$ BE binding by subtype-selective antagonists. A, inhibition by the  $\alpha_{1A}$ -selective antagonist (+)-niguldipine in membranes from HEK293 cells expressing Flag- $\alpha_{1A}$ , HA- $\alpha_{1B}$ , or both. B, inhibition by the  $\alpha_{1D}$ -selective antagonist BMY7378 in membranes from HEK293 cells expressing Flag- $\alpha_{1D}$ , HA- $\alpha_{1B}$ , or both. C, inhibition by the  $\alpha_{1D}$ -AR selective antagonist BMY7378 in membranes from HEK293 cells expressing Flag- $\alpha_{1D}$ , HA- $\alpha_{1A}$ , or both. Data are plotted as percentage of specific binding remaining at the indicated drug concentrations. Each point is the mean  $\pm$  S.E.M. from three to four different transfection experiments, each performed in duplicate.



pressed (Table 3). However, when  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs (which do not coimmunoprecipitate) were coexpressed, no significant increases were observed in the density of either subtype (Table 3). Compared with Flag- $\alpha_{1D}$ -ARs expressed alone, densitometric analysis of Western blots also detected an  $80 \pm 3.0\%$  increase in Flag- $\alpha_{1D}$ -AR total protein expression only when  $\alpha_{1B}$ -, and not  $\alpha_{1A}$ -ARs, were coexpressed (Fig. 9A). These results show that coexpression of subtypes that form heterodimers ( $\alpha_{1B} + \alpha_{1A}$  or  $\alpha_{1B} + \alpha_{1D}$ ) alters both receptor density and receptor protein levels, whereas coexpression of subtypes that do not form heterodimers ( $\alpha_{1A} + \alpha_{1D}$ ) does not. This coincidence raises the possibility that the observed alterations in receptor density may be a functional consequence of  $\alpha_1$ -AR heterodimerization.

**Increased Cell Surface Expression.** The data from the ligand binding and Western blot analysis suggested that the processing and/or trafficking of  $\alpha_{1D}$ -ARs might be regulated by coexpression with  $\alpha_{1B}$ -ARs. Therefore, we used a quantitative luminometer-based assay to directly determine whether the increase in  $\alpha_{1D}$ -AR expression when coexpressed with  $\alpha_{1B}$ -ARs was related to an increase in receptor surface expression. Interestingly, several laboratories have reported that  $\alpha_{1D}$ -ARs tend to be localized intracellularly and are poorly expressed on the cell surface (Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002). We determined surface expression of Flag- $\alpha_{1D}$ -ARs stably expressed in HEK293 cells ( $\sim 150$ – $250$  fmol/mg) alone and when coexpressed with HA- $\alpha_{1B}$ -ARs ( $\sim 300$ – $450$  fmol/mg), which were transiently expressed in these cells. As shown in Fig. 9B, there was a striking 10-fold increase in cell surface expression of  $\alpha_{1D}$ -ARs when coexpressed with  $\alpha_{1B}$ -ARs compared with  $\alpha_{1D}$ -ARs alone. These data reveal that coexpression of  $\alpha_{1B}$ -ARs facilitates efficient trafficking of  $\alpha_{1D}$ -ARs to the cell surface.

## Discussion

Although multiple  $\alpha_1$ -AR subtypes are known to coexist within tissues and cells, the functional importance of this coexistence remains unclear. All three subtypes are known to respond to the same neurotransmitters and couple to the same major signaling mechanisms, and responses to combinations of subtypes could therefore be additive, redundant, or synergistic (Graham et al., 1996; Zhong and Minneman, 1999; Varma and Deng, 2000). However, with the recent discovery that some GPCRs form dimers that alter their pharmacological and functional properties, the potential im-

plications of the coexistence of these subtypes must be examined more carefully. Therefore, in this study, we have examined the possibility that  $\alpha_1$ -AR subtypes homo- and heterodimerize into larger multiprotein species that may impact their pharmacological and functional properties.

Our laboratory recently reported that epitope-tagged  $\alpha_{1A}$ -,  $\alpha_{1B}$ -, and  $\alpha_{1D}$ -ARs form SDS-resistant homodimers and higher order oligomers when expressed in HEK293 cells. These SDS-resistant oligomers can be reduced to monomers under very strong denaturing conditions (6 M urea) (Vicentic et al., 2002), suggesting that noncovalent mechanisms are involved. This suggests that there is equilibrium between monomeric and oligomeric forms of the receptor, which can be altered by solubilization conditions. In the studies reported here, we provide direct evidence for homodimerization of all three subtypes in HEK293 cells by coimmunoprecipitation of  $\alpha_1$ -ARs with different epitope tags. Single subtypes tagged with either Flag- or HA- epitopes were coexpressed in HEK293 cells, solubilized, and immunoprecipitated with antibodies to each tag. Each subtype was found to coimmunoprecipitate with its alternatively tagged version, suggesting formation of homodimers that can be partially separated into monomers on an SDS gel under the denaturing conditions used. Interestingly, larger species with molecular masses characteristic of dimers or trimers were also observed in many of these experiments, although we do not know whether these contain the same or different tags. It seems that some homodimers are dissociated during preparation of samples for Western blots, whereas others are SDS-resistant and run as dimers after partial denaturation and electrophoresis.

Similar approaches were used to study heterodimerization between different  $\alpha_1$ -AR subtypes. Different subtypes with distinct epitope tags were coexpressed in HEK293 cells, solubilized, and immunoprecipitated with tag-specific antibodies. After electrophoresis and blotting, individual subtypes were detected using anti-tag antibodies. These experiments showed that  $\alpha_{1B}$ -ARs coimmunoprecipitate with either  $\alpha_{1A}$ - or  $\alpha_{1D}$ -ARs when expressed in the same cells. Because no such heterodimerization was observed when solubilized preparations from cells expressing single subtypes were mixed together, such coimmunoprecipitation does not seem to be a result of nonspecific hydrophobic interactions. Furthermore, heterodimerization does not occur via the association of partially solubilized receptors, because similar levels of heterodimers were still present after ultracentrifugation, which should pellet all partially solubilized proteins. Inter-

TABLE 4

Affinities of drugs in competing for specific  $^{125}$ IBE binding to  $\alpha_1$ -AR subtypes expressed alone and together in HEK293 cells

Differentially tagged  $\alpha_1$ -AR subtypes were expressed alone or together in HEK293 cells, membranes were harvested, and inhibition of specific  $^{125}$ IBE binding was determined for the drugs indicated.  $pK_I$  values were calculated by nonlinear regression of one- or two-site fits as described under *Materials and Methods*. Values are presented as mean  $\pm$  S.E.M. of three experiments performed in duplicate.

Subtypes Expressed	$pK_I$		(+)–Niguldipine		BMY 7378	
	NE	Prazosin	$pK_{I \text{ high}}$	$pK_{I \text{ low}}$	$pK_{I \text{ high}}$	$pK_{I \text{ low}}$
$\alpha_{1A}$ alone	$4.9 \pm 0.07$	$9.3 \pm 0.18$	$10.3 \pm 0.09$			$6.3 \pm 0.04$
$\alpha_{1B}$ alone	$5.2 \pm 0.04$	$9.7 \pm 0.07$		$7.1 \pm 0.08$		$6.6 \pm 0.04$
$\alpha_{1D}$ alone	$6.7 \pm 0.06$	$9.8 \pm 0.22$		$6.9 \pm 0.35$	$8.8 \pm 0.16$	
$\alpha_{1A} + \alpha_{1B}$	$5.2 \pm 0.05$	$9.3 \pm 0.04$	$10.2 \pm 0.05$	$7.3 \pm 0.09$	N.D.	N.D.
$\alpha_{1A} + \alpha_{1D}$	N.D.	$9.3 \pm 0.10$	N.D.	N.D.	$8.8 \pm 0.21$	$6.4 \pm 0.05$
$\alpha_{1B} + \alpha_{1D}$	$5.3 \pm 0.04$	$9.6 \pm 0.05$	N.D.	N.D.	$8.8 \pm 0.24$	$6.5 \pm 0.10$

N.D., not determined.

estingly, coimmunoprecipitation of  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs was not observed after coexpression, suggesting that heterodimer formation occurs only between specific combinations of subtypes.

Similar subtype-specific heterodimerization has been observed with other GPCRs, including opioid, serotonin, and somatostatin receptors (Bouvier, 2001; Rios et al., 2001). Coimmunoprecipitation studies show dimerization between  $\kappa$  and  $\delta$  but not  $\kappa$  and  $\mu$  opioid receptors (Jordan and Devi, 1999), whereas another study found  $\delta$  and  $\mu$  receptor heterodimers (George et al., 2000). Interestingly, recent work suggests that several different AR combinations, including  $\beta_1$ - and  $\beta_2$ -ARs (Lavoie et al., 2002) and  $\beta_1$ - and  $\alpha_{2A}$ -ARs (Xu et al., 2003), which coexist in many tissues, can also form

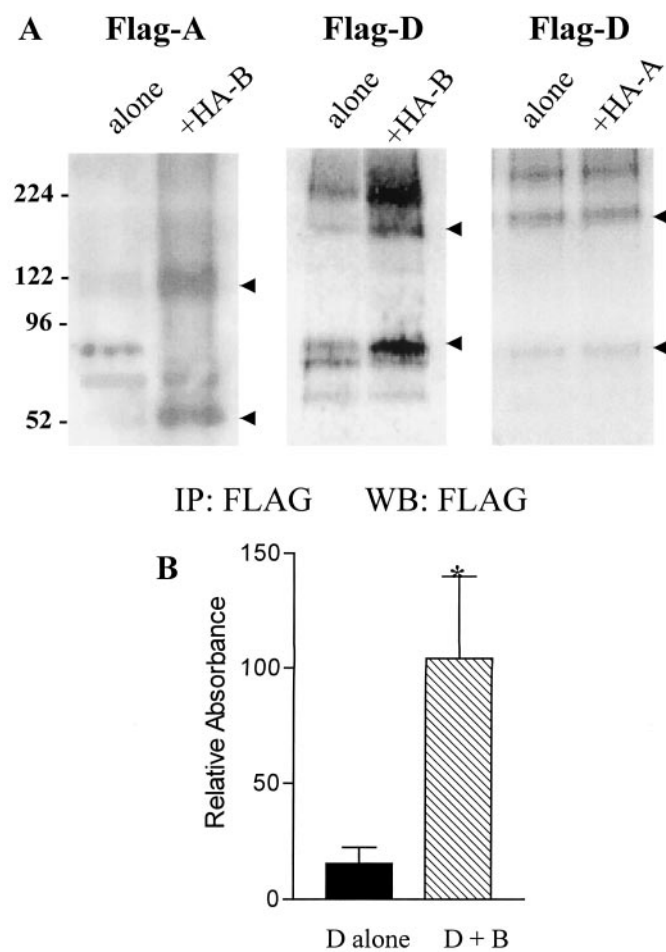
heterodimers that modulate receptor internalization and signaling. Taken together, these studies suggest that subtype-specific GPCR heterodimer formation may be widespread, with multiple functional consequences.

The effect of agonist activation on GPCR dimerization varies depending on the receptors involved. Agonist occupancy has been shown to increase, decrease or have no effect on dimerization of different GPCRs (Bouvier, 2001; Rios et al., 2001). We found that stimulation of intact cells with NE did not alter the levels of homodimers of any  $\alpha_1$ -AR subtype or the extent of  $\alpha_{1A}/\alpha_{1B}$  or  $\alpha_{1D}/\alpha_{1B}$  heterodimers and did not induce formation of  $\alpha_{1A}/\alpha_{1D}$  heterodimers. These results suggest that both homo- and heterodimerization of  $\alpha_1$ -ARs is constitutive and agonist-independent, similar to results obtained with  $\kappa$  opioid and M3 muscarinic receptor homodimers, and angiotensin1/bradykinin B2 heterodimers (Jordan and Devi, 1999; Jordan et al., 2000).

Mechanisms that have been implicated in GPCR dimerization include interactions between specific motifs within transmembrane domains, intracellular or extracellular loops, or N- or C-terminal extensions. For example, metabotropic glutamate receptors form dimers via covalent disulfide bonds in their very long extracellular N-termini, GABA<sub>B</sub> receptor C-termini play a role in GABA<sub>B</sub> receptor dimerization, and chimeric  $\alpha_{2A}$ -AR-m3 muscarinic receptors require the third intracellular loops for dimerization (Bouvier, 2001; Rios et al., 2001). We first examined whether the relatively long C-tails of  $\alpha_1$ -AR subtypes might be involved in dimerization by the use of C-truncated mutants. We found C-truncated receptors formed both homo- and heterodimers similar to those formed by full-length receptors, suggesting that their C-termini are not required for dimerization. Similar results have been observed with C-truncation mutants of  $\delta$  opioid and dopamine D3 receptors (Cvejic and Devi, 1997). We also examined the relatively long N terminus of  $\alpha_{1D}$ -ARs to determine whether it was required for  $\alpha_1$ -AR homo- or heterodimerization and found similar results. Mutated  $\alpha_{1D}$ -ARs lacking the N-terminal 79 aa formed SDS-resistant homodimers similar to full-length receptors (Pupo et al., 2003), and also formed heterodimers with  $\alpha_{1B}$ -, but not  $\alpha_{1A}$ -ARs, also similar to full-length  $\alpha_{1D}$ -ARs. These results suggest that dimerization of  $\alpha_1$ -ARs does not require C- and N-terminal domains, suggesting that  $\alpha_1$ -AR oligomerization may occur by a transmembrane domain-swapping model, as has been proposed previously for  $\beta_2$ -ARs (Hebert et al., 1996).

Expression of both homo- and heterodimers of  $\alpha_1$ -ARs on the cell surface was examined by use of a membrane-impermeant biotinylating reagent (Bai et al., 1998) and subsequent interaction with streptavidin. These data suggest that the observed  $\alpha_1$ -AR dimers and oligomers all seem to be expressed on the cell surface. This was particularly noticeable for  $\alpha_{1D}$ -ARs, which often have low expression levels in HEK293 cells, and in which the primary species seem to be homodimers and trimers (Vicentic et al., 2002). In fact, the pattern of expression of each subtype was similar for total and cell surface receptor expression, with mixtures of monomers and dimers predominating for  $\alpha_{1A}$ -, monomers for  $\alpha_{1B}$ -, and dimers and trimers for  $\alpha_{1D}$ -ARs. This suggests that homo- and hetero-oligomerization is not specifically confined to a single subcellular compartment.

One possible consequence of  $\alpha_1$ -AR dimerization might be changes in the pharmacological properties of the receptor



**Fig. 9.** A, Western blot analysis of  $\alpha_1$ -AR protein expression in membranes expressing single subtypes or mixtures of subtypes. Left, Flag- $\alpha_{1A}$ -ARs (Flag-A) expressed alone or in combination with HA- $\alpha_{1B}$ -ARs (HA-B). Center, Flag- $\alpha_{1D}$ -ARs (Flag-D) expressed alone or in combination with HA- $\alpha_{1B}$ -ARs. Right, Flag- $\alpha_{1D}$ -ARs expressed alone or in combination with HA- $\alpha_{1A}$ -ARs (HA-A). Membranes were solubilized, immunoprecipitated (IP) with anti-Flag affinity resin, and deglycosylated. Western blots (WB) were performed with HRP-conjugated anti-Flag antibody and are representative of three to four different transfection experiments. Arrows indicate apparent monomers and dimers of each receptor. B, cell surface expression of Flag- $\alpha_{1D}$ -ARs expressed alone or in combination with HA- $\alpha_{1B}$ -ARs was quantified via a luminometer-based assay as described under *Materials and Methods*. The values for each experiment are represented as arbitrary relative absorbance units. The bars and error bars represent the means  $\pm$  S.E.M. for three to six independent experiments. \*, significantly different ( $p < 0.05$ ), using an unpaired  $t$  test analysis, compared with the same subtype expressed alone.

binding sites. In fact, there is existing evidence for additional  $\alpha_1$ -AR subtypes that cannot be explained by the three known cloned subtypes (Endoh, 1996; Ford et al., 1996, 1997). Changes in pharmacological properties have been observed previously with opioid, somatostatin, and dopamine ( $D_2/D_3$ ) receptor heterodimers, which have novel ligand binding properties compared with subtypes expressed alone (Jordan and Devi, 1999; Scarselli et al., 2000). However, other GPCRs, such as 5-HT<sub>1B</sub>/5-HT<sub>1D</sub> serotonin receptor and opioid/adrenergic receptor ( $\delta$ -OR/ $\beta_2$ -AR) heterodimers, show no apparent differences in binding properties upon heterodimerization (Xie et al., 1999; Jordan et al., 2001). We used radioligand binding to examine the affinities of agonists and antagonists in membranes from cells expressing  $\alpha_1$ -AR subtypes alone or in different combinations. We found no changes in apparent  $K_D$  values for the nonsubtype selective antagonist prazosin when combinations of  $\alpha_1$ -AR subtypes were coexpressed, suggesting that heterodimerization does not explain the " $\alpha_{1L}$ " subtype previously observed in functional studies of smooth muscle (Ford et al., 1996; Muramatsu et al., 1998). However, we cannot rule out the possibility that the  $\alpha_{1L}$  subtype is not labeled by <sup>125</sup>IBE. We also examined the subtype-selective antagonists (+)-niguldipine ( $\alpha_{1A}$ ) and BMY7378 ( $\alpha_{1D}$ ). These drugs showed different  $K_I$  values for individual subtypes expressed alone as expected, but competition curves for these drugs in cells expressing combinations of subtypes were clearly biphasic. Nonlinear regression analysis showed that inhibition curves from cells coexpressing combinations of subtypes were best fit by a two-site model, with each of the sites characteristic of a single subtype expressed alone. For example, inhibition of specific <sup>125</sup>IBE binding in cells coexpressing  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs by (+)-niguldipine resulted in a two-site fit; one site had an affinity characteristic of the  $\alpha_{1A}$ -subtype and the other was characteristic of the  $\alpha_{1B}$ - subtype expressed alone. Similar results were obtained with the  $\alpha_{1D}$ -selective antagonist BMY7378 when  $\alpha_{1D}$ -ARs were coexpressed with other subtypes. These results suggest that  $\alpha_1$ -AR heterodimers do not display any unexpected pharmacological profiles but that their pharmacological properties seem to be caused by combinations of binding sites contributed by individual subtypes. Thus, heterodimerization of  $\alpha_1$ -ARs is not likely to account for the properties of novel subtypes that have been proposed in native tissues.

In contrast to the lack of effect of subtype coexpression on receptor pharmacological properties, the surface expression of  $\alpha_{1D}$ -ARs was strikingly increased upon coexpression with  $\alpha_{1B}$ -ARs, suggesting that  $\alpha_{1B}$ -ARs may assist in transporting  $\alpha_{1D}$ -ARs to the cell surface. Furthermore, coexpression of  $\alpha_{1B}$ -ARs with  $\alpha_{1A}$ - and  $\alpha_{1D}$ -ARs significantly increased the density of  $\alpha_{1A}$ - and  $\alpha_{1D}$ -AR binding sites and the amount of receptor protein observed on Western blots. The mechanisms by which such effects occur remain to be elucidated. It is interesting that heterodimerization is important in trafficking the GABA<sub>B</sub> receptor heterodimer to the cell surface, where the R2 subtype acts as a molecular chaperone and is essential for proper transport of the R1 subtype (Margeta-Mitrovic et al., 2001). In addition, dimerization of vasopressin and dopamine receptor C-truncated mutants with wild-type vasopressin V2 and dopamine D3 receptors prevents cell surface expression, supporting a role for dimerization in receptor processing and transport (Karpa et al., 2000; Zhu and

Wess, 1998). Our data raise the possibility that heterodimerization may enhance the efficiency of  $\alpha_1$ -AR processing and/or trafficking, resulting in increased receptor expression.

Interestingly, a number of previous studies have suggested that a large fraction of  $\alpha_{1D}$ -ARs are localized intracellularly, with a limited cell surface expression (Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002). For example,  $\alpha_{1D}$ -ARs fused with green fluorescent protein seem to be mainly cytosolic in HEK293 cells (Chalothorn et al., 2002). These findings support previous observations on low expression levels and poor coupling efficiencies of human  $\alpha_{1D}$ -ARs after heterologous expression in different cell lines (Perez et al., 1993; Esbenshade et al., 1995; Theroux et al., 1996; Taguchi et al., 1998), raising the possibility that this subtype cannot be efficiently folded and translocated to the surface membrane on its own. On the other hand, the  $\alpha_{1B}$  subtype has been found predominantly on the cell surface in most studies (Hirasawa et al., 1997; Daly et al., 1998; McCune et al., 2000; Chalothorn et al., 2002). Thus, our results suggest that coexpression of the surface-directed  $\alpha_{1B}$ -AR promotes surface trafficking of the  $\alpha_{1D}$  subtype, possibly by direct heterodimerization, although this remains to be further investigated.

It is known that rat cardiomyocytes express all three  $\alpha_1$ -AR subtypes (Noguchi et al., 1993, 1995; Zhong and Minneman, 1999) and that rat and human vascular smooth muscle cells express heterogeneous mixtures of subtypes (usually  $\alpha_{1B}$ - with  $\alpha_{1A}$ - or  $\alpha_{1D}$ -ARs) (Price et al., 1994; Graham et al., 1996; Piascik et al., 1997). Interestingly, studies in the peripheral vascular system show that  $\alpha_{1B}$ -AR mRNA is widespread, although this subtype does not seem to be involved in contraction (except in mesenteric resistance arteries) (Piascik et al., 1997). All three  $\alpha_1$ -AR subtypes are also expressed in several human brain regions, including cerebral cortex and cerebellum, where their functions may modulate sleep, arousal, and other important behavioral processes but remain largely unknown (Price et al., 1994; Graham et al., 1996). The widely observed coexistence of these subtypes in many different cells and tissues supports a potential role for heterodimerization in control of expression or trafficking of particular subtypes.

In summary, we used coimmunoprecipitation to show that differentially-tagged human  $\alpha_1$ -AR subtypes form agonist-independent homodimers that are expressed on the cell surface. We have also presented the first evidence for formation of heterodimers by certain combinations of  $\alpha_1$ -AR subtypes but not others. These heterodimers are constitutively present, are located on the cell surface, and require neither the extracellular amino terminus nor the intracellular carboxyl tail. Radioligand binding studies suggest that heterodimerization of  $\alpha_1$ -ARs does not change their affinities for norepinephrine or antagonists, suggesting that dimerization does not result in pharmacologically distinct subtypes. Coexpression with  $\alpha_{1B}$ -ARs significantly increases receptor density and total protein expression of both  $\alpha_{1A}$ - and  $\alpha_{1D}$ -AR subtypes and also significantly increases the proportion of  $\alpha_{1D}$ -ARs on the cell surface. These findings suggest that heterodimerization of human  $\alpha_1$ -AR subtypes are functionally relevant in the regulation of receptor processing and/or trafficking.



**Acknowledgments.** We thank Drs. Andre Pupo and Rebecca Wright for helpful discussions and experimental support and Mr. George Rogge for excellent technical support.

## References

- Bai M, Trivedi S, and Brown EM (1998) Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. *J Biol Chem* **273**:23605–23610.
- Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* **2**:274–286.
- Chalothorn D, McCune DF, Edelmann SE, Garcia-Cazarin ML, Tsujimoto G, and Piascik MT (2002) Differences in the cellular localization and agonist-mediated internalization properties of the  $\alpha_1$ -adrenoceptor subtypes. *Mol Pharmacol* **61**:1008–1016.
- Cvejic S and Devi LA (1997) Dimerization of the  $\delta$ -opioid receptor: implication for a role in receptor internalization. *J Biol Chem* **272**:26959–26964.
- Daly CJ, Milligan CM, Milligan G, Mackenzie JF and McGrath JC (1998) Cellular localization and pharmacological characterization of functioning  $\alpha_1$ -adrenoceptors by fluorescent ligand binding and image analysis reveals identical binding properties of clustered and diffuse populations of receptors. *J Pharmacol Exp Ther* **286**:984–990.
- Endoh M (1996) Cardiac  $\alpha_1$ (1)-adrenoceptors that regulate contractile function: subtypes and subcellular signal transduction mechanisms. *Neurochem Res* **21**:217–229.
- Esbenshade TA, Han C, Murphy TJ, and Minneman KP (1993) Comparison of  $\alpha_1$ -adrenoreceptor subtypes and signal transduction in SK-N-MC and NB41A3 neuronal cell lines. *Mol Pharmacol* **44**:76–86.
- Esbenshade TA, Hirasawa A, Tsujimoto G, Tanaka T, Yano J, Minneman KP, and Murphy TJ (1995) Cloning of the human  $\alpha_{1D}$ -adrenoreceptor and inducible expression of three human subtypes in SK-N-MC cells. *Mol Pharmacol* **47**:977–985.
- Ford AP, Arredondo NF, Blue DR Jr, Bonhaus DW, Jasper J, Kava MS, Lesnick J, Pfister JR, Shieh IA, Vimont RL, et al. (1996) RS-17053 (*N*-(2-(2-cyclopropylmethoxyphenoxy)ethyl)-5-chloro- $\alpha$ , $\alpha$ -dimethyl-1*H*-indole-3-ethanamine hydrochloride), a selective  $\alpha_{1A}$ -adrenoceptor antagonist, displays low affinity for functional  $\alpha_1$ -adrenoceptors in human prostate: implications for adrenoceptor classification. *Mol Pharmacol* **49**:209–215.
- Ford AP, Daniels DV, Chang DJ, Gever JR, Jasper JR, Lesnick JD, and Clarke DE (1997) Pharmacological pleiotropism of the human recombinant  $\alpha_{1A}$ -adrenoceptor: implications for  $\alpha_1$ -adrenoceptor classification. *Br J Pharmacol* **121**:1127–1135.
- George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, and O'Dowd BF (2000) Oligomerization of  $\mu$ - and  $\delta$ -opioid receptors. Generation of novel functional properties. *J Biol Chem* **275**:26128–26135.
- Gether U, Lin S, and Kobilka BK (1995) Fluorescent labeling of purified  $\beta_2$  adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* **270**:28268–28275.
- Graham RM, Perez DM, Hwa J, and Piascik MT (1996) Alpha 1-adrenergic receptor subtypes. Molecular structure, function and signaling. *Circ Res* **78**:737–749.
- Han CD and Minneman KP (1991) Interaction of subtype-selective antagonists with  $\alpha$ -adrenoreceptor binding sites in rat tissues. *Mol Pharmacol* **40**:531–538.
- Hebert TE, Moffett S, Morello JP, Loiselle TP, Bichet DG, Barret C, and Bouvier M (1996) A peptide derived from a  $\beta_2$ -adrenoreceptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* **271**:16384–16392.
- Heldin CH (1995) Dimerization of cell surface receptors in signal transduction. *Cell* **80**:213–223.
- Hirasawa A, Shibata K, Horie K, Takei Y, Obika K, Tanaka T, Muramoto N, Takagaki K, Yano J, and Tsujimoto G (1995) Cloning, functional expression and tissue distribution of human  $\alpha_1$ -adrenoceptor splice variants. *FEBS Lett* **363**:256–260.
- Hirasawa A, Sugawara T, Awaji T, Tsumaya K, Ito H, and Tsujimoto G (1997) Subtype-specific differences in subcellular localization of  $\alpha_1$ -adrenoceptors: chloroethylclonidine preferentially alkylates the accessible cell surface  $\alpha_1$ -adrenoceptors irrespective of the subtype. *Mol Pharmacol* **52**:764–770.
- Johnson RD and Minneman KP (1986) Characterization of alpha 1-adrenoceptors which increase cyclic AMP accumulation in rat cerebral cortex. *Eur J Pharmacol* **129**:293–305.
- Jordan BA, Cvejic S, and Devi LA (2000) Opioids and their complicated receptor complexes. *Neuropsychopharmacology* **23**:S5–S18.
- Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature (Lond)* **399**:697–700.
- Jordan BA, Trapaidze N, Gomes I, Nivarthi R, and Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc Natl Acad Sci USA* **98**:343–348.
- Karpa KD, Lin R, Kabbani N, and Levenson R (2000) The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3–D3nf interaction causes mislocalization of D3 receptors. *Mol Pharmacol* **58**:677–683.
- Lavoie C, Mercier JF, Salahpour A, Umapathy D, Breit A, Villeneuve LR, Zhu WZ, Xiao RP, Lakatta EG, Bouvier M, et al. (2002)  $\beta_1/\beta_2$ -adrenoreceptor heterodimerization regulates  $\beta_2$ -adrenoreceptor internalization and ERK signaling efficacy. *J Biol Chem* **277**:35402–35410.
- Lei B, Zhang Y, and Han C (2002) Changes in mRNA expression induced by sustained noradrenaline stimulation are different for  $\alpha_1$ -adrenoceptor subtypes in HEK293 cells. *Clin Exp Pharmacol Physiol* **29**:1084–1090.
- Maggio R, Vogel Z, and Wess J (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular “cross-talk” between G-protein-linked receptors. *Proc Natl Acad Sci USA* **90**:3103–3107.
- Margeta-Mitrovic M, Jan YN, and Jan LY (2001) Function of GB1 and GB2 subunits in G-protein coupling of GABA(B) receptors. *Proc Natl Acad Sci USA* **98**:14649–14654.
- McCune DF, Edelmann SE, Olges JR, Post GR, Waldrop BA, Waugh DJ, Perez DM, and Piascik MT (2000) Regulation of the cellular localization and signaling properties of the  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenoceptors by agonists and inverse agonists. *Mol Pharmacol* **57**:659–666.
- Michelotti GA, Price DT, and Schwinn DA (2000) Alpha 1-adrenergic receptor regulation: basic science and clinical implications. *Pharmacol Ther* **88**:281–309.
- Muramatsu I, Murata S, Isaka M, Piao HL, Zhu J, Suzuki F, Miyamoto S, Oshita M, Watanabe Y, and Taniguchi T (1998) Alpha1-adrenoceptor subtypes and two receptor systems in vascular tissues. *Life Sci* **62**, 1461–1465.
- Noguchi H, Muraoka R, Kigoshi S, and Muramatsu I (1993) Alpha 1-adrenoceptor subtype involved in the positive inotropic response to phenylephrine in rat atria. *Eur J Pharmacol* **240**:291–293.
- Noguchi H, Muraoka R, Kigoshi S, and Muramatsu I (1995) Pharmacological characterization of alpha 1-adrenoceptor subtypes in rat heart: a binding study. *Br J Pharmacol* **114**:1026–1030.
- Perez DM, DeYoung MB, and Graham RM (1993) Coupling of expressed  $\alpha_{1B}$ - and  $\alpha_{1D}$ -adrenergic receptor to multiple signaling pathways is both G protein and cell type specific. *Mol Pharmacol* **44**:784–795.
- Piascik MT, Hrometz SL, Edelmann SE, Guarino RD, Hadley RW, and Brown RD (1997) Immunocytochemical localization of the  $\alpha_{1B}$  adrenergic receptor and the contribution of this and the other subtypes to vascular smooth muscle contraction: analysis with selective ligands and antisense oligonucleotides. *J Pharmacol Exp Ther* **283**:854–868.
- Price DT, Lefkowitz RJ, Caron MG, Berkowitz D, and Schwinn DA (1994) Localization of mRNA for three distinct  $\alpha_1$ -adrenergic receptor subtypes in human tissues: implications for human  $\alpha$ -adrenergic physiology. *Mol Pharmacol* **45**:171–175.
- Pupo AS, Uberti MA, and Minneman KP (2003) N-terminal truncation of human  $\alpha_1(1D)$ -adrenoceptors increases expression of binding sites but not protein. *Eur J Pharmacol* **462**:1–8.
- Ramarao CS, Denker JM, Perez DM, Gaivin RJ, Riek RP, and Graham RM (1992) Genomic organization and expression of the human  $\alpha_{1B}$ -adrenergic receptor. *J Biol Chem* **267**:21936–21945.
- Rios CD, Jordan BA, Gomes I, and Devi LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* **92**:71–87.
- Robeva AS, Woodard R, Luthin DR, Taylor HE, and Linden J (1996) Double tagging recombinant A1- and A2A-adenosine receptors with hexahistidine and the FLAG epitope. Development of an efficient generic protein purification procedure. *Biochem Pharmacol* **51**:545–555.
- Rokosh DG, Stewart AF, Chang KC, Bailey BA, Karliner JS, Camacho SA, Long CS and Simpson PC (1996)  $\alpha_1$ -Adrenergic receptor subtype mRNAs are differentially regulated by  $\alpha_1$ -adrenergic and other hypertrophic stimuli in cardiac myocytes in culture and in vivo. Repression of  $\alpha_{1B}$  and  $\alpha_{1D}$  but induction of  $\alpha_{1C}$ . *J Biol Chem* **271**:5839–5843.
- Scarselli M, Armogida M, Chiacchio S, DeMontis MG, Colzi A, Corsini GU, and Maggio R (2000) Reconstitution of functional dopamine D(2)s receptor by co-expression of amino- and carboxyl-terminal receptor fragments. *Eur J Pharmacol* **397**:291–296.
- Schwinn DA (1994) Adrenergic receptors: unique localization in human tissues. *Adv Pharmacol* **31**:333–341.
- Schwinn DA, Johnston GI, Page SO, Mosley MJ, Wilson KH, Worman NP, Campbell S, Fidock MD, Furness LB, Parry-Smith DJ, et al. (1995) Cloning and pharmacological characterization of human  $\alpha_1$  adrenergic receptors: sequence corrections and direct comparison with other species homologues. *J Pharmacol Exp Ther* **272**:134–142.
- Taguchi K, Yang M, Goepel M, and Michel MC (1998) Comparison of human  $\alpha_1$ -adrenoceptor subtype coupling to protein kinase C activation and related signaling pathways. *Naunyn-Schmiedeberg's Arch Pharmacol* **357**:100–110.
- Theroux TL, Esbenshade TA, Peavy RD, and Minneman KP (1996) Coupling efficiencies of human  $\alpha_1$ -adrenergic receptor subtypes: titration of receptor density and responsiveness with inducible and repressible expression vectors. *Mol Pharmacol* **50**:1376–1387.
- Varma DR and Deng XF (2000) Cardiovascular  $\alpha_1$ -adrenoceptor subtypes: functions and signaling. *Can J Physiol Pharmacol* **78**:267–292.
- Vicentic A, Robeva A, Rogge G, Uberti M, and Minneman KP (2002) Biochemistry and pharmacology of epitope-tagged  $\alpha_1$ -adrenergic receptor subtypes. *J Pharmacol Exp Ther* **302**:58–65.
- Xie Z, Lee SP, O'Dowd BF, and George SR (1999) Serotonin 5-HT1B and 5-HT1D receptors form homodimers when expressed alone and heterodimers when co-expressed. *FEBS Lett* **456**:63–67.
- Xu J, He J, Castleberry AM, Balasubramanian S, Lau AG, and Hall RA (2003) Heterodimerization of  $\alpha_2A$ - and  $\beta_1$ -adrenergic receptors. *J Biol Chem* **278**:10770–10777.
- Zhong H and Minneman KP (1999) Alpha1-adrenoceptor subtypes. *Eur J Pharmacol* **375**:261–276.
- Zhu X and Wess J (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37**:15773–15784.

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