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Heterodimers of α_{1B}- and α_{1D}-Adrenergic Receptors Form a Single Functional Entity

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ABSTRACT

Heterologous expression of α_{1D}-adrenergic receptors (α_{1D}-ARs) in most cell types results in intracellular retention and little or no functionality. We showed previously that heterodimerization with α_{1B}-ARs promotes surface localization of α_{1D}-ARs. Here, we report that the α_{1B}-/α_{1D}-AR interaction has significant effects on the pharmacology and signaling of the receptors, in addition to the effects on trafficking described previously. Upon coexpression of α_{1B}-ARs and epitope-tagged α_{1D}-ARs in both human embryonic kidney 293 and DDT_1MF-2 cells, α_{1D}-AR binding sites were not detectable with the α_{1D}-AR selective antagonist 8-[2-(4-(2-methoxyphenyl)piperazin-1-yl)ethyl]-8-azaspiro[4,5]decane-7,9-dione (BMY 7378), despite the ability to detect α_{1D}-AR protein using confocal microscopy, immunoprecipitation, and a luminometer cell-surface assay. However, the α_{1B}-AR-selective mutant F18A conotoxin showed a striking biphasic inhibition in α_{1B}/α_{1D}-AR-expressing cells, revealing that α_{1D}-ARs were expressed but did not bind BMY 7378 with high affinity. Studies of norepinephrine-stimulated inositol phosphate formation showed that maximal responses were greatest in α_{1B}/α_{1D}-AR-coexpressing cells. Stable coexpression of an uncoupled mutant α_{1B}-AR (Δ12) with α_{1D}-ARs resulted in increased responses to norepinephrine. However, Schild plots for inhibition of norepinephrine-stimulated inositol phosphate formation showed a single low-affinity site for BMY 7378. Thus, our findings suggest that α_{1B}/α_{1D}-AR heterodimers form a single functional entity with enhanced functional activity relative to either subtype alone and a novel pharmacological profile. These data may help to explain why α_{1D}-ARs are often pharmacologically undetectable in native tissues when they are coexpressed with α_{1B}-ARs.

An emerging paradigm in the field of pharmacology is that G-protein-coupled receptors (GPCRs) can form homo- and heterodimers, resulting in the formation of unique multiprotein complexes that have altered trafficking, signaling, and pharmacological properties (Milligan et al., 2004; Terrillon and Bouvier, 2004; Prinster et al., 2005). In fact, recent data have raised the possibility that homodimerization may be a ubiquitous process that is required for the proper expression of GPCRs (Canals et al., 2004; Kaykas et al., 2004; Salahpour et al., 2004). A growing number of reports implicating a clinical role for GPCR dimerization in opiate analgesia (Jordan and Devi, 1999), human immunodeficiency virus infection (Rodriguez-Frade et al., 2004), and vitreoretinopathy (Kaykas et al., 2004) highlight the need to continue characterizing the mechanisms and properties of novel GPCR dimers.

Numerous studies have now shown that GPCR heterodimerization is essential for proper expression and function of $GABA_B$ (Marshall et al., 1999), taste (Nelson et al., 2001), olfactory (Hague et al., 2004b), and α_{1D}-adrenergic receptors (ARs) (Hague et al., 2004c). The most convincing and thoroughly studied example to date of GPCR heterodimerization involves the formation of functional $GABA_B$ receptors. It is now clear that $GABA_BR1$ and $GABA_BR2$ must heterodimerize to ensure trafficking of $GABA_B$ receptors to the cell surface (Kaupmann et al., 1998; Marshall et al., 1999) at least partially through the masking of an endoplasmic reticulum (ER) retention signal located in the carboxyl-terminal tail of $GABA_BR1$ receptors (Margeta-Mitrovic et al., 2000). In addition, the formation of sweet taste receptors requires heterodimerization of T1R2 and T1R3 receptors (Nelson et al., 2001), and the M71 mouse olfactory receptor can achieve surface expression and become functional when heterodimerized with the β_2-AR (Hague et al., 2004b). In previous studies, we showed that α_{1D}-AR heterodimerization

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; AR, adrenergic receptor; NE, norepinephrine; InsP, inositol phosphate; GFP, green fluorescent protein; HEK, human embryonic kidney; HA, hemagglutinin; ER, endoplasmic reticulum; WT, wild type; PI, phosphatidylinositol; 5-MU, 5-methylurapidil; PBS, phosphate-buffered saline; BE 2254, 2-β(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; BMY 7378, 8-[2-(4-(2- methoxyphenyl)piperazin-1-yl)ethyl]-8-azaspiro[4,5]decane-7,9-dione.

with α_{1B}-ARs was required to promote surface expression of the intracellularly retained α_{1D}-AR (Hague et al., 2004c). These examples provide compelling evidence for GPCR heterodimerization in regulating GPCR cellular localization. However, with a handful of exceptions, such as δ- and κ-opioid (Jordan and Devi, 1999), D2 and D3 dopamine (Maggio et al., 2003), and α_{2A}-/β_1-adrenergic receptors (Xu et al., 2003), few examples of receptor heterodimerization causing significant pharmacological changes have been reported to date.

One longstanding mystery in the α_1-AR field has been the inability to detect α_{1D}-AR binding sites in intact tissues with the α_{1D}-AR-selective antagonist BMY 7378 (Yang et al., 1997, 1998), despite the fact that α_{1D}-AR mRNA is as widely expressed throughout the body as mRNA for the α_{1A}-AR and α_{1B}-AR subtypes (Rokosh et al., 1994; Alonso-Llamazares et al., 1995; Scofield et al., 1995). Previous studies have suggested that α_{1D}-AR mRNA may only be translated in response to specific stimuli, such as a loss of other α_1-AR subtypes (Turnbull et al., 2003) or hypertension (Ibarra et al., 2000). On the other hand, α_{1D}-AR mRNA may be widely translated, but α_{1D}-AR ligand binding may be masked or may exhibit altered properties in certain tissues. It has long been apparent that the α_{1D}-AR is the most poorly coupled of all α_1-ARs (Theroux et al., 1996) and that one possible reason could be that it functions poorly without a binding partner, such as the α_{1B}-AR. We report in this study that α_{1D}-ARs coexpressed with α_{1B}-ARs are undetectable with BMY 7378. Using immunochemical, biochemical, and pharmacological approaches, we found that α_{1D}-/α_{1B}-AR heterodimers act as a single entity with novel pharmacological properties, and each receptor subunit contributes a specific functional component to the complex.

Materials and Methods

Materials. Materials were obtained from the following sources: cDNAs for the wild-type human α_{1A}-AR (Hirasawa et al., 1993) and human α_{1D}-AR C-terminally tagged GFP constructs in pEGFP-N3 (Xu et al., 1999) were generously provided by Dr. Gozoh Tsujimoto (National Children's Hospital, Tokyo, Japan), human α_{1B}-AR cDNA (Ramarao et al., 1992) was a gift from Dr. Dianne Perez (Cleveland Clinic, Cleveland, OH), and human α_{1D}-AR cDNA was cloned in our laboratory (Esbenshade et al., 1995); FLAG/GFP-tagged human α_{1D}-ARs and $\Delta^{1-79}\alpha_{1D}$-ARs were created previously in our laboratory (Vicentic et al., 2002; Hague et al., 2004a). Hamster $\Delta 12\alpha_{1B}$-AR in pCMV was a gift from Dr. Myron Toews (University of Nebraska Medical Center, Omaha, NE); ρ-T1A and F18A mutants were a gift from Dr. Richard Lewis (Xenome Ltd., Queensland, Australia); HEK293 and DDT_1MF-2 cells were from American Type Culture Collection (Manassas, VA); 5-methylurapidil, niguldipine, BMY 7378, (−)-norepinephrine bitartrate, Dowex 1 Resin, horseradish peroxidase-conjugated anti-Flag M2 antibody, and bovine serum albumin were from Sigma-Aldrich (St. Louis, MO); [*myo*-^{3}H]inositol was from American Radiolabeled Chemicals (St. Louis, MO); Lipofectamine 2000 transfection reagent, fetal bovine serum, and penicillin/streptomycin were from Invitrogen (Carlsbad, CA); enzyme-linked immunosorbent assay enhanced chemiluminescence was from Pierce Chemical (Rockford, IL); Vectashield mounting medium was from Vector Laboratories (Burlingame, CA); and Dulbecco's modified Eagle's medium was from Cellgro-Mediatech (Herndon, VA).

Cell Culture and Transfection. HEK293 and DDT_1MF-2 cells were propagated in Dulbecco's modified Eagle's medium with sodium pyruvate supplemented with 10% heat inactivated fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere with 5% CO_2. Confluent plates were subcultured at a ratio of 1:5 for transfection. HEK293 and DDT_1MF-2 cells were transfected with 10 μg of DNA of each construct for 3 h using Lipofectamine 2000 transfection reagent, and cells were used for experimentation 48 to 72 h after transfection. Stable transfection of receptors was obtained by selection with 400 μg/ml G418 (pcDNA3.1, pDT, and pEGFP vectors) or 200 μg/ml hygromycin (pREP4 vector).

Luminometer-Based Surface-Expression Assay. DDT_1MF-2 cells were split into poly(D-lysine)-coated 35-mm dishes and incubated with horseradish peroxidase-conjugated M2-anti-FLAG antibody in blocking buffer, and cell-surface luminescence was determined using a method described previously (Hague et al., 2004c).

Laser Confocal Microscopy. Cells were grown on sterile coverslips, fixed for 30 min with 2% paraformaldehyde in 0.1 M phosphate buffer, washed, mounted, and scanned with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss GmbH, Heidelberg, Germany) as described previously (Hague et al., 2004c). For detecting GFP, fluorescein isothiocyanate fluorescence was excited using an argon laser at a wavelength of 488 nm, and the absorbed wavelength was detected for 510 to 520 nm for GFP.

Immunoprecipitation/Immunoblotting. DDT_1MF-2 cells expressing FLAG-α_{1D} GFP ARs were harvested by scraping in ice-cold phosphate-buffered saline (PBS) and washed by repeated centrifugation and homogenization. Cell lysates were solubilized, immunoprecipitated with anti-FLAG M2 affinity resin, and probed using anti-FLAG M2 monoclonal antibodies as described previously (Uberti et al., 2003).

Radioligand Binding. Confluent 150-mm plates were washed with PBS (20 mM $NaPO_4$ and 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were collected by centrifugation, homogenized with a Polytron homogenizer (Kinematica, Basel, Switzerland), centrifuged at 30,000*g* for 20 min, and resuspended in PBS. Radioligand binding sites were measured by saturation analysis of specific binding of the α_1-adrenergic receptor antagonist radioligand ^{125}I-BE 2254 (20–800 pM). Nonspecific binding was defined as binding in the presence of 10 μM phentolamine. The pharmacological specificity of radioligand binding sites was determined by displacement of ^{125}I-BE 2254 (50–70 pM) by prazosin, 5-MU, niguldipine, NE, F18A, and BMY 7378, and data were analyzed using nonlinear regression.

Measurement of [^{3}H]InsP Formation. Accumulation of [^{3}H]inositol phosphates (InsPs) was determined in confluent 96-well plates by a protocol described previously (Hague et al., 2004c). After prelabeling, medium containing [*myo*-^{3}H]inositol was removed, and 100 μl of Krebs-Ringer bicarbonate buffer (120 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, and 0.029 mM Na_2EDTA) containing 10 mM LiCl was gently added to each well. Cells were incubated with or without 100 μM NE for 60 min. For studies using BMY 7378, antagonist was added to cells for 30 min before the addition of agonist. The reaction was stopped by the addition of 100 μl of 20 mM formic acid, and samples were sonicated for 10 s. Samples were subjected to anion exchange chromatography to isolate [^{3}H]InsPs, which were quantified by scintillation counting.

Data Analysis and Statistics. Radioligand binding and [^{3}H]InsP formation data were calculated as means ± S.E.M. and statistical comparisons used GraphPad Prism Software (GraphPad Software Inc., San Diego, CA). Schild plots were calculated according to the method described originally by Arunlakshana and Schild (1959).

Results

α_{1D}-AR Binding Sites Are Undetectable with BMY 7378 in DDT_1MF-2 Cells Expressing α_{1D}-AR Protein. We have shown previously that intracellular α_{1D}-ARs require heterodimerization with α_{1B}-ARs to promote their expression at the cell surface (Hague et al., 2004c). Because DDT_1MF-2 cells endogenously express α_{1B}-ARs at approximately 300 to

400 fmol/mg of protein, we stably transfected these cells with FLAG-α_{1D}-GFP ARs to use as a model system for functional and pharmacological characterization of α_{1B}-/α_{1D}-AR heterodimers. As expected, confocal microscopy (Fig. 1A) and a luminometer-based cell-surface assay (Fig. 1B) indicated that α_{1D}-ARs were quantitatively expressed at the cell surface. In support of these findings, immunoblotting for FLAG revealed that FLAG-α_{1D}-GFP protein was expressed (Fig. 1C), suggesting that a significant number of α_{1D}-ARs were expressed at the cell surface. Although Western blots are only semiquantitative, careful titration of N-truncated α_{1D}-AR binding site expression with the density of signal on Western blots suggests that this should translate into ~600 fmol/mg of protein of α_{1D}-AR binding sites (data not shown). Therefore, we expected to see corresponding increases in the α_1-AR B_{max} and the appearance of α_{1D}-AR binding sites. However, in saturation binding experiments, we found no significant differences in receptor expression levels between untransfected and α_{1D}-AR expressing DDT$_1$MF-2 cells (Fig. 1D). In addition, α_{1D}-AR binding sites were undetectable in ^{125}I-BE 2254 competition binding experiments using the α_{1D}-AR selective antagonist BMY 7378. Only a single population of α_{1B}-AR low-affinity binding sites consistent with previously observed values at α_{1B}-ARs (Goetz et al., 1995) was observed in both wild-type and α_{1D}-AR transfected cell lines. Thus, our confocal and biochemical data suggested that α_{1D}-ARs were expressed at the plasma membrane after transfection into DDT$_1$MF-2 cells. However, our findings from radioligand binding experiments suggested that α_{1D}-ARs were not detectable pharmacologically.

α_{1D}-AR Binding Sites Are Undetectable with BMY 7378 in HEK293 Cells Coexpressing α_{1D}-/α_{1B}-ARs. From our data obtained in DDT$_1$MF-2 cells, we hypothesized that our inability to detect α_{1D}-AR binding sites might be caused by low α_{1D}-AR expression levels, despite the fact that the Western blots suggested that they should be easily detectable. Therefore, we chose to switch to HEK293 cells as a model to characterize α_{1B}-/α_{1D}-AR heterodimers, because in previous studies, we have found that extremely high receptor expression levels can be obtained using this cell line (Uberti et al., 2003; Hague et al., 2004a,c). To create a HEK293 cell line stably coexpressing α_{1B}-/α_{1D}-ARs, we first transfected HA-α_{1B}-ARs in the pREP4 vector and selected with hygromycin until only resistant cells remained. After selection, we confirmed the presence of HA-α_{1B}-ARs by performing ^{125}I-BE 2254 competition binding experiments using BMY 7378, which detected a homogenous population of low-affinity binding sites (Fig. 2A). FLAG-α_{1D}-GFP ARs were then stably transfected into HEK293 cells alone or into HEK293 cells stably expressing HA-α_{1B}-ARs. ^{125}I-BE 2254 competition

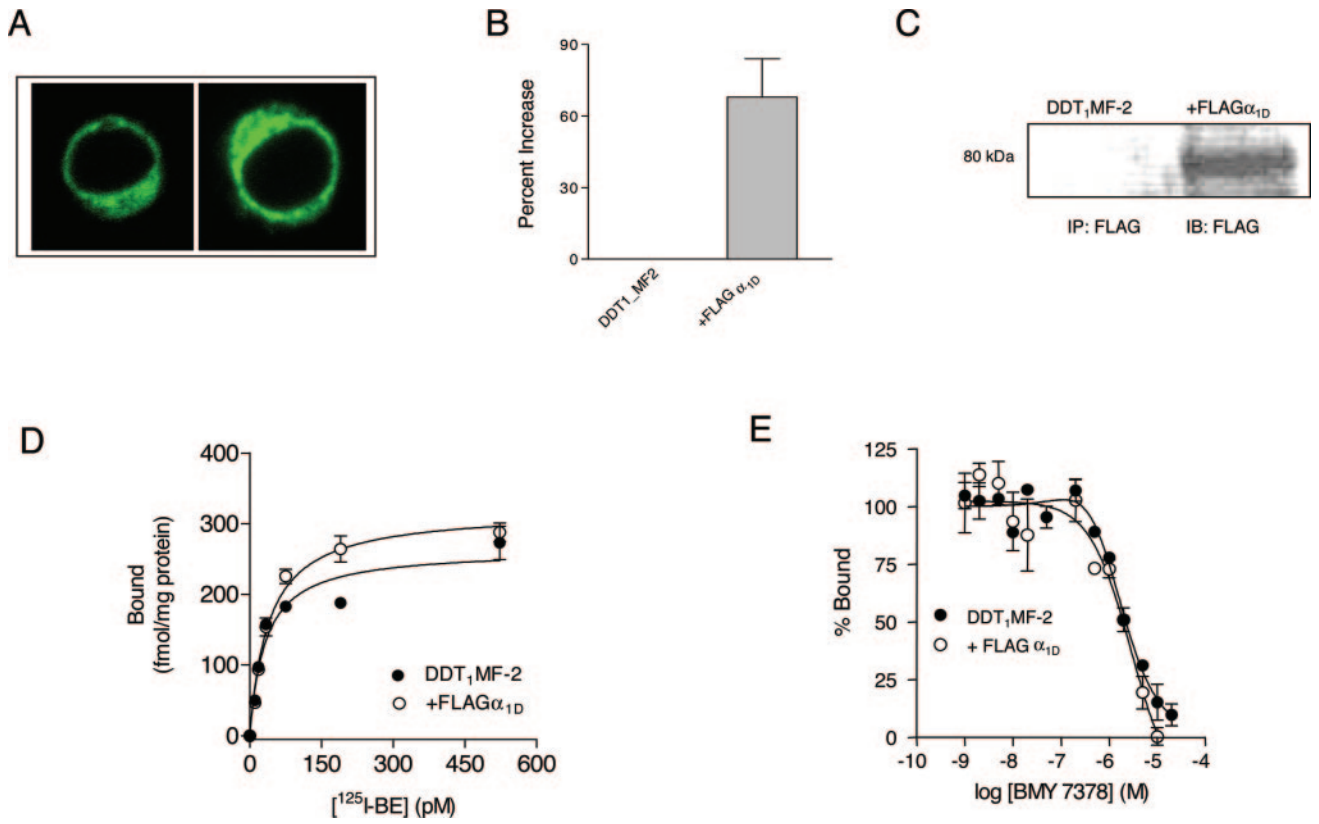

Fig. 1. Heterologous expression of α_{1D}-ARs with native hamster α_{1B}-ARs in DDT$_1$MF-2 cells. A, confocal imaging of FLAG-α_{1D}-GFP ARs stably expressed in DDT$_1$MF-2 cells. Cells were fixed and excited using an argon-neon laser (488 nm) as described under *Materials and Methods*. B, Cell-surface expression of α_{1D}-ARs in DDT$_1$MF-2 cells. Cell-surface expression of FLAG-α_{1D}-GFP ARs was detected using a luminometer-based assay, as described under *Materials and Methods*. The values for each experiment are represented as the percentage of absorbance over untransfected DDT$_1$MF-2 cells. The data are expressed as mean ± S.E.M. of three independent experiments. C, immunoprecipitation of FLAG-α_{1D}-GFP ARs stably expressed in DDT$_1$MF-2 cells. Cells were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG antibodies as described under *Materials and Methods*. D, ^{125}I-BE 2254 saturation binding analysis of untransfected (●) or stably transfected DDT$_1$MF-2 cells expressing FLAG-α_{1D}-GFP ARs (○). Data are expressed as mean ± S.E.M. from three individual experiments performed in duplicate. E, BMY 7378 competition binding analysis of untransfected (●) or stably transfected DDT$_1$MF-2 cells expressing FLAG-α_{1D}-GFP ARs (○). Data are expressed as mean ± S.E.M. from four individual experiments performed in duplicate.

binding with BMY 7378 identified a single population of high-affinity BMY 7378 binding sites in HEK293 cells expressing FLAG-α_{1D}-GFP alone. However, similar to our observations in DDT$_1$MF-2 cells, BMY 7378 detected only a single population of low-affinity binding sites in HEK293 cells coexpressing FLAG-α_{1D}-GFP and HA-α_{1B}-ARs (Fig. 2A). A screen of a panel of α_1-AR-selective antagonists (Table 1) provided no further evidence for the presence of α_{1D}-AR binding sites. The α_{1A}-AR-selective antagonists 5-MU and niguldipine recognized a low-affinity binding site, and the nonselective ligands prazosin, BE 2254, and NE all bound within the range of affinities reported previously. Therefore, to determine whether α_{1D}-ARs were expressed in this cell line using an alternative method, HEK293 cells coexpressing FLAG-α_{1D}-GFP and HA-α_{1B}-ARs were fixed on coverslips and examined using confocal microscopy. As shown in Fig. 2B, FLAG-α_{1D}-GFP ARs were quantitatively expressed at the plasma membrane in this cell line, which was in direct contrast to our radioligand binding data suggesting that α_{1D}-ARs were not expressed. Finally, Western blots from cell lysates were then immunoprecipitated and run on SDS gels and were compared with Western blots from HEK293 cells expressing N-truncated α_{1D}-ARs at approximately 450 fmol/mg of protein (Fig. 2C). The results from our biochemical and confocal studies indicated that α_{1D}-ARs were present when coexpressed with α_{1B}-ARs and should have been forming functional binding sites, yet our pharmacological data indicate that they were not.

To ensure that α_{1D}-ARs would be expressed at high levels, we used a previously generated HEK293 cell subclone expressing a high density of wild-type (WT) human α_{1D}-AR binding sites (B_{max} = 920 fmol/mg of protein; data not shown). With this high α_{1D}-AR expression level, we hypothesized that overexpressing α_{1B}-ARs in this cell line would still allow for easy detection of α_{1D}-AR binding sites. WT human α_{1A}- or α_{1B}-ARs in the pREP4 vector were then transfected into the high-expression WT α_{1D}-AR subclone and selected using hygromycin. B_{max} values were then determined using ^{125}I-BE 2254 saturation binding. As shown in Fig. 3A, HEK293 cells transfected with empty pREP4 alone had no significant increase in α_{1D}-AR expression levels (B_{max} = 1204 fmol/mg of protein), whereas the B_{max} value in α_{1A}-/α_{1D}-AR-coexpressing cells increased to 1845 fmol/mg of protein (Table 2). It is interesting that the B_{max} in α_{1B}-/α_{1D}-AR-coexpressing cells was unchanged at 1070 fmol/mg of protein, which was not significantly different from the B_{max} in HEK293 cells expressing α_{1D}-ARs alone. From these findings, we hypothesized that detection of α_{1D}-AR binding sites with BMY 7378 would be possible in this α_{1B}-/α_{1D}-AR coexpression cell line. As shown in Fig. 3B, ^{125}I-BE 2554 competition binding with BMY 7378 revealed the expected result of a heterogeneous population of high- and low-affinity binding sites in α_{1A}-/α_{1D}-AR-coexpressing HEK293 cells and a single population of high-affinity binding sites in α_{1D}-AR-expressing cells with empty pREP4 vector. However, BMY 7378 recognized only a single population of low-affinity binding sites in α_{1B}-/α_{1D}-AR-coexpressing cell lines. These findings have three possible explanations: either 1) all previously expressed α_{1D}-ARs had been replaced with transfected α_{1B}-ARs; 2) α_{1B}-/α_{1D}-ARs were both expressed and formed a heterodimer that has low affinity for BMY 7378; or 3) the presence of α_{1B}-ARs alters the structure of α_{1D}-ARs such that they cannot bind ^{125}I-BE 2554.

A ρ-T1A Mutant Peptide Reveals Multiple Binding Sites in HEK293 Cells Coexpressing α_{1B}-/α_{1D}-ARs. We have previously characterized a conotoxin peptide ρ-T1A isolated from the sea snail to be an α_{1B}-AR subtype-selective antagonist that acts noncompetitively at the α_{1B}- and com-

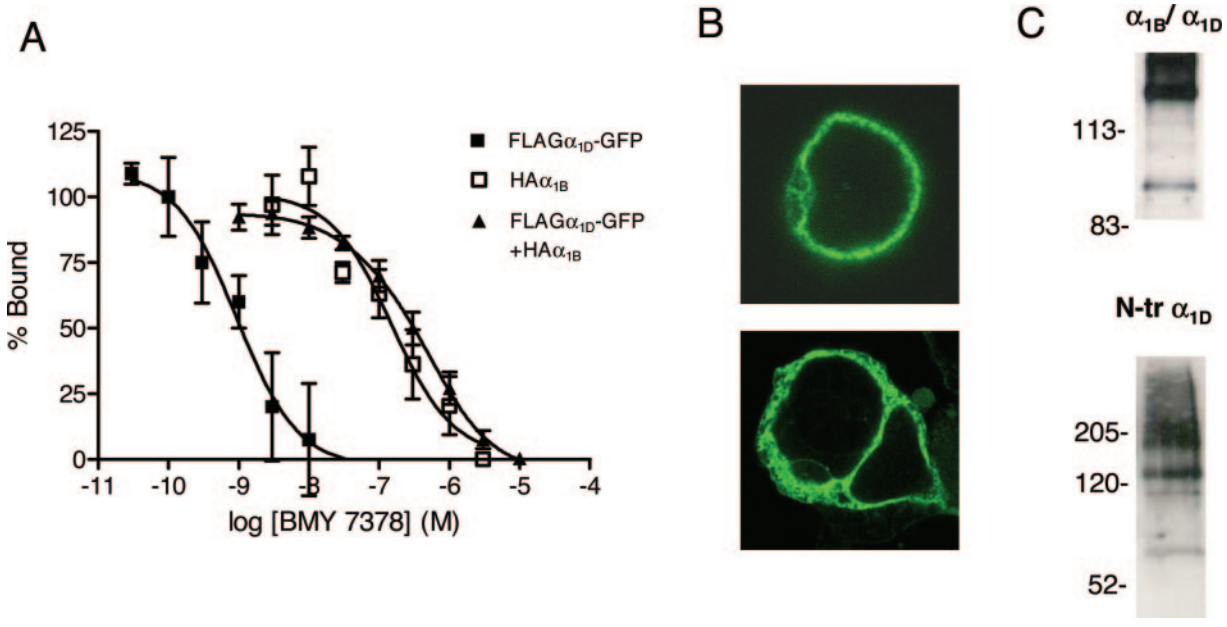

Fig. 2. BMY 7378 recognizes a single binding site in HEK293 cells coexpressing α_{1B}- and α_{1D}-ARs. A, ^{125}I-BE 2254 competition radioligand binding was used to determine BMY 7378 binding affinities in HEK293 cells expressing FLAG-α_{1D}-GFP (■), HA-α_{1B} (□), or coexpressing FLAG-α_{1D}-GFP and HA-α_{1B} ARs (▲). Data are the mean of four independent experiments performed in duplicate and are expressed as mean ± S.E.M. B, confocal imaging of fluorescein isothiocyanate fluorescence in HEK293 cells coexpressing FLAG-α_{1D}-GFP and HA-α_{1B} ARs. Cells were fixed and excited with an argon-neon laser at 488 nm as described under *Materials and Methods*. C, to semiquantitatively estimate the density of α_{1D}-AR binding sites expected, we performed immunoprecipitation and Western blotting for the FLAG epitope and compared it to N-truncated (Ntr) α_{1D}-ARs, which form binding sites and localize to the cell surface.

petitively at the α_{1A}- and α_{1D}-AR subtypes (Chen et al., 2004). Taken from its differential modes of inhibition at the α_1-AR subtypes, it is likely that ρ-T1A binds to regions of the α_1-ARs other than the conserved catecholamine binding pocket. ρ-T1A is a noncompetitive inhibitor of α_{1B}-ARs but competitively inhibits α_{1D}-ARs (Chen et al., 2004). Figure 4A demonstrates that this peptide is in fact a competitive inhibitor in HEK293 cells coexpressing α_{1B}/α_{1D}-ARs. An alanine mutant of ρ-T1A, F18A, demonstrated significant selectivity between the α_{1B}- and α_{1D}-AR subtypes (~20-fold). Therefore, we performed competition binding experiments using F18A in the hope that it would distinguish between α_{1B}-AR and α_{1D}-AR binding sites. As shown in Fig. 4, we found that F18A recognized a single population of low-affinity binding sites in HEK293 cells expressing α_{1D}-ARs and a heterogeneous population of low-affinity binding sites in the α_{1A}-/α_{1D}-AR coexpressing HEK293 cells (Fig. 4). F18A unexpectedly recognized a mixture of high- and low-affinity binding sites in HEK293 cells cotransfected with α_{1B}-/α_{1D}-ARs (Fig. 3C). Approximately 66% of the binding sites were high affinity, with pIC_{50} values of −9.0, whereas the remaining 34% of the binding sites were low affinity, with pIC_{50} values of −7.0 (Table 2). Therefore, these data suggest that F18A can recognize multiple binding sites in HEK293 cells cotransfected with α_{1D}-AR and α_{1B}-AR receptor subtypes, whereas BMY 7378 recognizes only a single population of low-affinity binding sites.

TABLE 1

Log K_I values for α_1-AR-selective ligands determined from ^{125}I-BE 2254 competition binding studies performed in HEK293 cells stably expressing HA-α_{1B}-ARs alone or together with FLAG-α_{1D}-GFP ARs

Drug	HA-α_{1B}-AR	HA-α_{1B}-AR/ FLAG-α_{1D}-GFP
Prazosin	−9.3 ± 0.09	−9.8 ± 0.03
Niguldipine	−6.9 ± 0.04	−6.9 ± 0.02
5-MU	N.D.	−6.8 ± 0.03
BMY 7378	−6.1 ± 0.09	−6.4 ± 0.02
NE	−5.0 ± 0.09	−4.9 ± 0.02
^{125}I-BE 2254	−10.4 ± 0.07	−9.9 ± 0.06

N.D., not determined.

Coexpression of N-Truncated α_{1D}-ARs with α_{1B}-ARs Reveals α_{1D}-AR Binding Sites. Previous reports from our laboratory (Hague et al., 2004a,c) and others (McCune et al., 2000; Chalothorn et al., 2002) have demonstrated that α_{1D}-ARs are primarily intracellular when expressed alone but can be trafficked to the cell surface upon N-terminal truncation (Hague et al., 2004a) or coexpression with α_{1B}-ARs (Hague et al., 2004c). However, the data shown above suggest that although α_{1B}-ARs can heterodimerize and traffic α_{1D}-ARs to the cell surface, this does not result in an increase in binding site density or α_{1D}-AR binding sites. Therefore, one potential interpretation of these findings is that α_{1B}-/α_{1D}-AR heterodimers form a single receptor complex, resulting in the formation of a novel binding pocket that binds BMY 7378 with low affinity. To test this hypothesis, we coexpressed N-truncated (Δ^{1-79}) α_{1D}-ARs with α_{1B}-ARs in HEK293 cells. Previous work revealed that N-truncated α_{1D}-ARs are capable of forming heterodimers with α_{1B}-ARs (Uberti et al., 2003) but do not require α_{1B}-AR coexpression for trafficking to the cell surface (Hague et al., 2004a). Thus, we predicted that coexpressing $\Delta^{1-79}\alpha_{1D}$-ARs with α_{1B}-ARs may result in the expression of a mixed population of high- and low-affinity BMY 7378 binding sites, because N-truncated α_{1D}-ARs do not depend on α_{1B}-ARs for cell-surface trafficking like the wild-type α_{1D}-ARs do. We created multiple HEK293 cell lines stably expressing $\Delta^{1-79}\alpha_{1D}$-GFP ARs and determined their receptor expression levels using ^{125}I-BE 2254 saturation

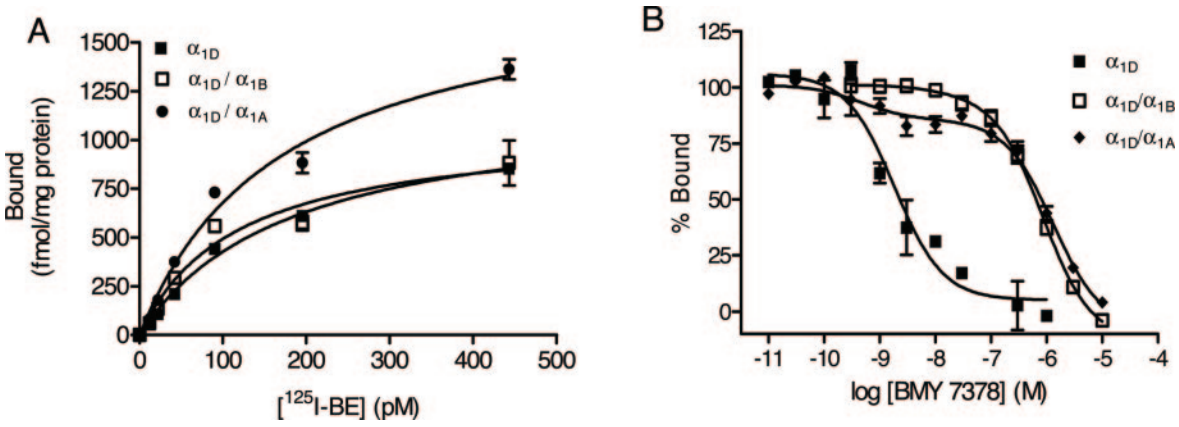

Fig. 3. BMY 7378 recognizes a single population of binding sites in α_{1B}-AR/α_{1D}-AR-coexpressing cells. WT α_{1A}-AR and α_{1B}-ARs were stably transfected into HEK293 cells expressing α_{1D}-ARs as described under *Materials and Methods*. Cell membranes expressing WT α_{1D}-ARs alone (■) or coexpressed with WT α_{1A}-ARs (♦) or WT α_{1B}-ARs (□) were prepared and used for ^{125}I-BE 2254 saturation binding (A) and competition binding experiments with BMY 7378 (B). Data are the means of six to nine independent experiments performed in duplicate and are expressed as mean ± S.E.M.

TABLE 2

B_{max} and pK_I or pIC_{50} values determined from ^{125}I-BE 2254 saturation and competition radioligand binding assays in HEK293 cells expressing α_{1D}-ARs

Subtypes Expressed	B_{max}	BMY 7378		%$_{high}$	F18A		%$_{high}$
		p$K_{I\ high}$	pK_{low}		$pIC_{50\ high}$	$pIC_{50\ low}$	
	fmol/mg						
α_{1D} + pREP4	1204 ± 46	−8.7 ± 0.1		100	−7.4 ± 0.03		100
α_{1D} + HAα_{1B}	1070 ± 117		−6.1 ± 0.01	0	−9.0 ± 0.04	−7.0 ± 0.07	66
α_{1D} + HAα_{1A}	1845 ± 19	−9.3 ± 0.2	−5.9 ± 0.04	14	−7.6 ± 0.26	−5.9 ± 0.09	27

binding. As shown in Fig. 5A, approximately 1200 to 1400 fmol/mg of protein of Δ^{1-79} α_{1D}-GFP ARs were expressed in each cell line, with the majority of these receptors expressed at the cell surface, as determined by confocal microscopy (Fig. 5B). HA-α_{1B}-ARs in the pREP4 vector were then transfected into each cell line and selected with hygromycin to produce HEK293 cells coexpressing α_{1B}-/$\Delta^{1-79}\alpha_{1D}$-GFP ARs. It is interesting that cell line 1 demonstrated no significant increase in receptor density (B_{max} = 1126 fmol/mg of protein; Fig. 5A), yet BMY 7378 distinguished a mixed population of high- (58%) and low-affinity (42%) binding sites (Fig. 5B; Table 3). In direct contrast, cell line 2 demonstrated a ~2-fold increase in receptor density (B_{max} = 2902 fmol/mg of protein) (Fig. 5A), but BMY 7378 recognized only a single population of low-affinity binding sites (Fig. 5B; Table 3). Therefore, these findings suggest that at nonsaturating levels of α_{1B}-AR ex-

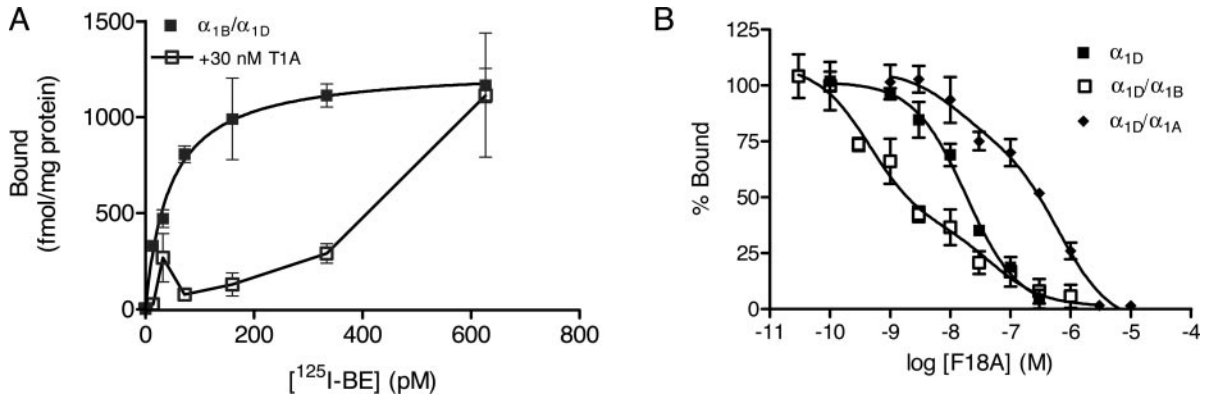

Fig. 4. The conotoxin peptides ρ-T1A and F18A recognizes multiple binding sites in HEK293 cells coexpressing α_{1B}- and α_{1D}-ARs. WT α_{1A}-AR and α_{1B}-ARs were stably transfected into HEK293 cells expressing α_{1D}-ARs as described under *Materials and Methods*. A, ^{125}I-BE 2254 saturation binding analysis was performed in the absence (■) or presence of 30 nM ρ-T1A (□) in HEK293 membranes coexpressing α_{1B}-/α_{1D}-ARs. Data are the means of three independent experiments performed in duplicate and are expressed as mean ± S.E.M. B, cell membranes expressing WT α_{1D}-ARs alone (■) or coexpressed with WT α_{1A}-ARs (♦) or WT α_{1B}-ARs (□) were prepared and used for ^{125}I-BE 2254 competition binding experiments with F18A. Data are the means of six to nine independent experiments performed in duplicate and are expressed as mean ± S.E.M.

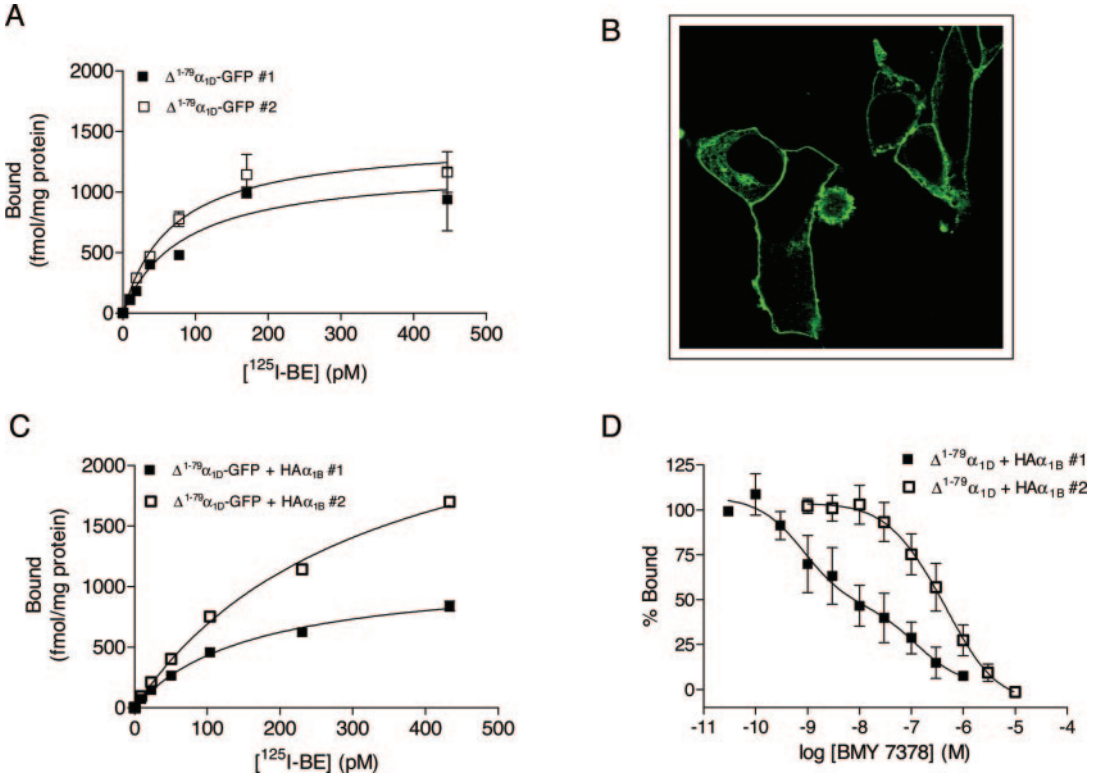

Fig. 5. N-truncated α_{1D}-ARs binding sites are detectable when coexpressed with α_{1B}-ARs. A, GFP-tagged Δ^{1-79} α_{1D}-ARs were stably transfected into HEK293 cells and were subjected to saturation binding analysis using ^{125}I-BE 2254. Data are the means of four independent experiments performed in duplicate and are expressed as mean ± S.E.M. B, confocal image of HEK293 cells stably expressing GFP-tagged Δ^{1-79} α_{1D}-ARs. Cells were excited with an argon-neon laser (488 nm) as described under *Materials and Methods*. C, saturation binding analysis of HEK293 cells coexpressing Δ^{1-79} α_{1D}- and α_{1B}-ARs. HEK293 cells stably expressing GFP-tagged Δ^{1-79} α_{1D}-ARs were stably transfected with HA-α_{1B}-ARs and subjected to saturation binding analysis using ^{125}I-BE 2254. Cell lines 1 (■) and 2 (□) represent separate HA-α_{1B}-AR transfections. Data are the means of three independent experiments performed in duplicate and are expressed as mean ± S.E.M. D, HEK293 cells coexpressing Δ^{1-79} α_{1D}- and α_{1B}-ARs were subjected to ^{125}I-BE 2254 competition binding to determine BMY 7378 affinities. Cell lines 1 (■) and 2 (□) represent separate HA-α_{1B}-AR transfections and are the same cell lines used in C. Data are the means of three independent experiments performed in duplicate and are expressed as mean ± S.E.M.

pression, there is a mixed population of α_1-ARs expressed: $\Delta^{1-79}\alpha_{1D}$-GFP ARs alone (high-affinity BMY 7378 binding sites), α_{1B}-ARs alone, and α_{1B}-ARs heterodimerized with $\Delta^{1-79}\alpha_{1D}$-GFP ARs (low-affinity BMY 7378 binding sites). However, at saturating levels of α_{1B}-AR expression, only low-affinity BMY 7378 binding sites are found, which include α_{1B}-AR and α_{1B}-/$\Delta^{1-79}\alpha_{1D}$-GFP AR heterodimers.

α_{1B}-/α_{1D}-AR Heterodimers Have Increased Maximal Responses. The data shown above suggest that α_{1B}- and α_{1D}-ARs form heterodimeric complexes that are characterized with low-affinity binding for the α_{1D}-AR-selective antagonist BMY 7378. We next examined the contributions of each α_1-AR subtype to the overall signaling of the α_{1B}-/α_{1D}-AR complex. In previous studies, we found that α_{1B}-/α_{1D}-AR heterodimerization increased the rate of α_{1D}-AR internalization and the maximal levels of intracellular Ca^{2+} mobilization in response to NE stimulation but resulted in only minor increases in maximal PI hydrolysis (Hague et al., 2004c). To further characterize the role of each α_1-AR subtype in the heterodimeric complex, we performed cell-surface assays to determine the rate of α_{1B}-AR internalization. We found that stimulation of α_{1B}-ARs transiently transfected in HEK293 cells resulted in a 40 to 50% loss in the number of cell-surface receptors after 30 min, with no further increase after 60 min (Fig. 6A). Coexpressing α_{1B}-ARs with α_{1D}-ARs resulted in no significant difference in the rate of α_{1B}-AR internalization, suggesting that the α_{1B}-/α_{1D}-AR heterodimer is equally susceptible to agonist-induced endocytosis.

To further examine the functional importance of this heterodimer, we used our HEK293 cell lines stably coexpressing WT α_{1B}-/α_{1D}-ARs to generate NE concentration-response curves for InsP formation to determine whether there were any differences in agonist potency or intrinsic activity. As shown in Fig. 6B, NE had greater intrinsic activity in cells coexpressing WT α_{1B}-/α_{1D}-ARs than those expressing α_{1B}- or α_{1D}-ARs alone, or in mixtures of cells expressing α_{1B}- and α_{1D}-ARs alone, suggesting WT α_{1B}-/α_{1D}-AR heterodimers act as a high-efficacy receptor complex.

α_{1B}- and α_{1D}-ARs Have Distinct Functional Roles within the Heterodimeric Complex. To eliminate any functional responses produced by α_{1B}-AR stimulation, we created HEK293 cell lines stably coexpressing WT α_{1D}-ARs and an α_{1B}-AR mutant missing three amino acids in the N-terminal portion of the third intracellular loop, which is uncoupled from functional responses ($\Delta12\alpha_{1B}$-ARs) but is still capable of promoting cell-surface expression of α_{1D}-ARs (Hague et al., 2004c). Similar to our observations in α_{1B}-/α_{1D}-AR-coexpressing cells, BMY 7378 recognized a single population of low-affinity binding sites in cells expressing α_{1D}-/$\Delta12\alpha_{1B}$-ARs (K_I = −6.27, Fig. 7B), and NE functional responses were significantly greater than those in cells expressing α_{1D}-ARs alone (Fig. 7A). Because $\Delta12\alpha_{1B}$-ARs do not couple to functional responses (Fig. 7A), we hypothesized that BMY 7378 would inhibit NE functional responses in α_{1D}-/$\Delta12\alpha_{1B}$-AR-coexpressing cells with high affinity. To investigate this, we incubated HEK293 cells stably coexpressing α_{1D}-/$\Delta12\alpha_{1B}$-ARs with increasing concentrations of BMY 7378 for 30 min and generated NE concentration-response curves for InsP formation. Only at high concentrations (1, 3, 10, and 30 μM) did BMY 7378 cause parallel shifts to the right in the NE-concentration curve (Fig. 7C). Schild regression analysis of the data (Fig. 7D) revealed a functional affinity constant of −6.05 ± 0.6 with slope not significantly different from unity. This functional affinity constant for BMY 7378 is characteristic of α_{1B}-AR (6.0) and not α_{1D}-AR (8.5). Therefore, these data provide strong additional evidence that BMY 7378 inhibits NE functional responses at α_{1B}-/α_{1D}-AR heterodimers with low affinity.

TABLE 3

B_{max} and pK_I values determined from ^{125}I-BE 2254 saturation and competition radioligand binding assays in HEK293 cells expressing $\Delta^{1-79}\alpha_{1D}$-GFP ARs

Cell Line	B_{max}	Change in B_{max}	BMY 7378		%$_{high}$
			p$K_{I\,high}$	p$K_{I\,low}$	
	fmol/mg	*%*			
$\Delta^{1-79}\alpha_{1D}$ 1	1209 ± 163		−8.7 ± 0.05		100
+ HAα_{1B}	1126 ± 36	↓ 7	−9.1 ± 0.13	−6.9 ± 0.22	58
$\Delta^{1-79}\alpha_{1D}$ 2	1434 ± 104		−8.6 ± 0.04		100
+ HAα_{1B}	2902 ± 146	↑ 102		−6.4 ± 0.03	0

Discussion

From this and previous studies, it is now clear that α_1-ARs undergo subtype-specific heterodimerization in heterologous systems. α_{1B}-ARs can heterodimerize with both α_{1A}-ARs (Stanasila et al., 2003; Uberti et al., 2003) and α_{1D}-ARs (Uberti et al., 2003; Hague et al., 2004c), whereas α_{1A}-ARs are unable to heterodimerize with α_{1D}-ARs (Uberti et al., 2003). Heterodimerization of α_{1B}/α_{1D}-ARs promotes cell-surface expression of intracellularly localized α_{1D}-ARs. To determine the functional significance of this interaction, we further characterized the pharmacological and functional properties of α_{1B}-/α_{1D}-AR heterodimers.

We were surprised to find that in cell lines stably coexpressing both α_{1B}- and epitope-tagged α_{1D}-ARs, α_{1D}-AR expression could be detected using immunoprecipitation and confocal fluorescence microscopy but could not be detected pharmacologically with the α_{1D}-AR-selective antagonist BMY 7378 in radioligand binding experiments. Comparison of Western blots using the epitope tags suggested that significant numbers of α_{1D}-AR binding sites (500 fmol/mg of protein or greater) should have been present. In addition, no increase in binding-site density was observed in comparison with cells expressing either subtype alone, suggesting that α_{1B}-/α_{1D}-AR heterodimers form a single binding site, or that the presence of α_{1B}-ARs alters α_{1D}-ARs such that they cannot bind ^{125}I-BE 2254. However, a mutant of the conotoxin ρ-T1A (F18A) showed biphasic inhibition in cells coexpressing α_{1B}-/α_{1D}-ARs. When coexpressing functionally uncoupled α_{1B}-ARs with full-length WT α_{1D}-ARs, the α_{1D}-AR-selective antagonist BMY 7378 inhibited functional responses to NE with a low affinity, suggesting these two receptors are acting as individual components of a heterodimeric complex. These findings strongly suggest that α_{1B}- and α_{1D}-ARs heterodimerize to form a single functional entity.

One of the most surprising findings of this study was that BMY 7378 was unable to detect α_{1D}-AR binding sites when coexpressed with α_{1B}-ARs, despite the fact that α_{1D}-ARs were detectable with immunoprecipitation and confocal techniques. In fact, when α_{1B}-ARs were stably overexpressed in an HEK293 cell subclone expressing α_{1D}-ARs at very high levels, the number of binding sites did not change, but the pharmacology of BMY 7378 shifted from a single high- to

single low-affinity population of sites. This is particularly interesting given that α_{1D}-ARs are largely undetectable with BMY 7378 in most intact tissues (Yang et al., 1997, 1998), despite the fact that α_{1D}-AR mRNA is as widely expressed as the mRNAs for the α_{1A}-AR and α_{1B}-AR subtypes (Rokosh et al., 1994; Alonso-Llamazares et al., 1995; Scofield et al., 1995). For example, in a recent study, mRNA for all three α_1-AR subtypes was detectable in rat submandibular gland cells. However, BMY 7378 detected only a single population of low-affinity binding sites in radioligand binding experiments (Bockman et al., 2004), which is consistent with our findings suggesting that coexpression of α_{1B}- and α_{1D}-ARs results in the masking of high-affinity α_{1D}-AR binding sites. In addition, the affinity of BMY 7378 in inhibiting phenylephrine-mediated contraction was found to be significantly increased in isolated carotid arteries from α_{1B}-AR knockout mice (Deighan et al., 2005), and phenylephrine-stimulated increases in left ventricular-developed pressure were only inhibited by BMY 7378 in α_{1A}-/α_{1B}-AR double knockout mice (Turnbull et al., 2003). These unusual findings could be explained by a model wherein the knockout of α_{1B}-ARs from native tissues results in the unmasking of α_{1D}-AR binding sites with high affinity for BMY 7378, which would be predicted from the results of our cellular studies reported here.

There is an emerging role for dimerization in the biosynthesis and maturation of GPCRs (Bulenger et al., 2005), and it is possible that the expression of the α_{1B}-AR with the α_{1D}-AR relieves a block on the intracellular or post-translational processing of the latter which allows it to be expressed on the cell surface or other aspects of protein maturational processing. Although we do not yet have evidence for such processes, further studies are likely to clarify whether this is important in this interaction.

Rat (Piascik et al., 1995) and mouse (Yamamoto and Koike, 2001) aortas have long been the preferred model system to study α_{1D}-AR functional responses. From our previous studies demonstrating that α_{1B}-AR heterodimerization with α_{1D}-ARs promotes cell-surface expression (Hague et al., 2004c), we expected that α_{1B}-AR knockout mice would have diminished α_{1D}-AR-mediated functional responses. In fact, studies of phenylephrine-stimulated contraction of aorta from α_{1B}-AR knockout mice have given conflicting results. In the original characterization of these mice, aortic contraction was significantly diminished (Cavalli et al., 1997). However, a subsequent study reported that aortic contraction is essentially unaltered in α_{1B}-AR knockout mice (Daly et al., 2002). We reported recently that β_2-ARs can also promote α_{1D}-AR cell-surface expression, and unlike α_{1D}-/α_{1B}-AR heterodimers, they maintain a high affinity for BMY 7378 (Uberti et al., 2005). Therefore, one possibility is that both α_{1B}-ARs and β_2-ARs may contribute to α_{1D}-AR function in mouse aorta.

Accumulating evidence now suggests that each receptor within a GPCR heterodimer is responsible for a particular component of the signaling complex. Several examples of this can be observed in the class III family of GPCRs, including the $GABA_B$ (Jones et al., 1998; Kaupmann et al., 1998) and taste (Nelson et al., 2001) receptors. It is noteworthy that within the $GABA_B$ receptor heterodimer, the $GABA_BR2$ subunit is responsible for promoting surface expression of the $GABA_BR1$ (Jones et al., 1998; Kaupmann et al., 1998; Margeta-Mitrovic et al., 2001) by masking an ER retention motif in the $GABA_BR1$ C-terminal tail (Calver et al., 2000; Margeta-Mitrovic et al., 2000). Once properly assembled, the $GABA_BR1$ subunit seems to be primarily responsible for agonist binding, whereas the $GABA_BR2$ subunit couples to G-proteins (Margeta-Mitrovic et al., 2001). It is interesting that we have found that the α_{1B}-/α_{1D}-AR heterodimer is functionally similar to the $GABA_B$ receptor heterodimer. The α_{1B}-AR serves to promote cell-surface expression of the α_{1D}-AR (Hague et al., 2004c), possibly by masking an ER

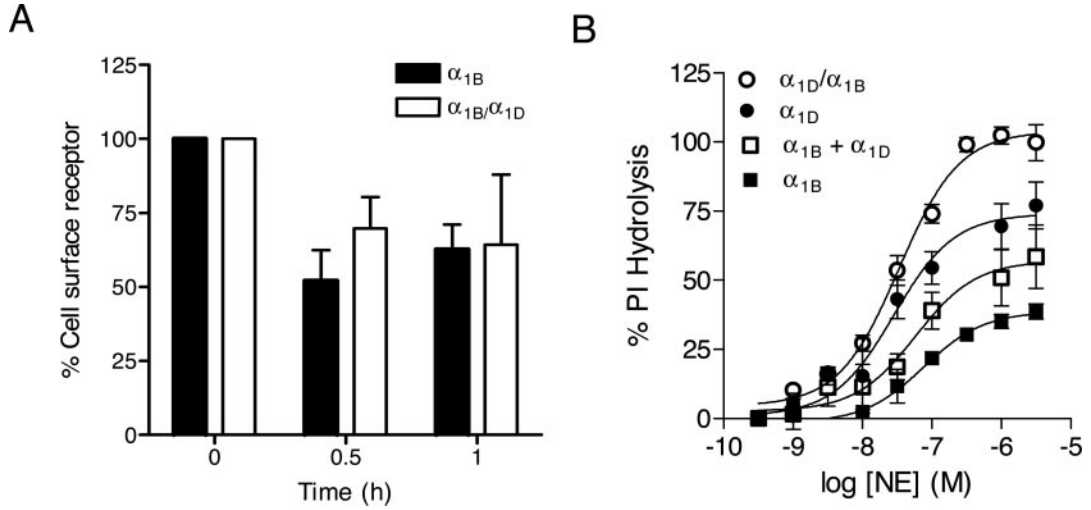

Fig. 6. α_{1B}-/α_{1D}-AR heterodimers have increased norepinephrine maximal responses. A, coexpression of α_{1B}-/α_{1D}-ARs does not effect the internalization parameters of α_{1B}-ARs. Cell-surface expression of FLAG-α_{1B}-ARs was determined using a fluorescent luminometer assay as described under *Materials and Methods*. HEK293 cells expressing FLAG-α_{1B}-ARs alone and in combination with HA-α_{1D}-ARs were stimulated with 10 μM NE for 30 and 60 min. Data are expressed a mean ± S.E.M. of three experiments performed in triplicate. B, coexpression of α_{1B}-/α_{1D}-ARs results in increased NE maximal responses. HEK293 cells expressing α_{1D}-ARs alone (●), α_{1B}-ARs alone (■), coexpressed α_{1B}-/α_{1D}-ARs (○), or an equal mixture of cells expressing α_{1B}-ARs and α_{1D}-ARs alone (□) were incubated with [*myo*-^{3}H]inositol for 24 h. Cells were then stimulated with increasing concentrations of NE for 1 h and were assayed for [^{3}H]InsP production as described under *Materials and Methods*. Data are expressed as the percentage of PI hydrolysis, with 100% stimulation equal to the level attained in cells coexpressing α_{1B}-/α_{1D}-ARs. Data are the means of three individual experiments performed in duplicate.

retention motif in the α_{1D}-AR N terminus (Pupo et al., 2003; Hague et al., 2004a; Petrovska et al., 2005). In addition, it seems that within the $\Delta12\alpha_{1B}$-AR/α_{1D}-AR heterodimer, the $\Delta12\alpha_{1B}$-AR is primarily responsible for binding ligand, whereas the α_{1D}-AR couples to G protein activation, but whether this is true for wild-type α_{1B}-ARs remains to be determined. Individual receptor subunits acting as distinct components within a heterodimer complex have also been shown previously to occur with heterodimers consisting of H1 histamine and α_{1B}-ARs (Carrillo et al., 2003), β_2-ARs and δ-opioid receptors (Jordan et al., 2001), β_2-ARs and α_{2A}-ARs (Xu et al., 2003), and β_2-ARs and β_3-ARs (Breit et al., 2004). Taken together, these findings suggest that GPCR heterodimers form functional complexes with distinct pharmacological and signaling properties in which each receptor subunit may be responsible for specific functions. Most of these studies have been done, by necessity, in heterologous expression systems in which receptor density is difficult to control. The functional significance of class I GPCR heterodimers has been demonstrated recently in vivo for opioid receptors using a heterodimer-selective agonist (Waldhoer et al., 2005), consistent with the hypothesis that these complexes occur in native tissues.

The existence of α_{1B}-/α_{1D}-AR heterodimers may seem perplexing, especially because α_{1B}-ARs are functional when expressed alone. We have found that α_{1B}-/α_{1D}-AR heterodimers stimulate greater maximal NE responses relative to α_{1B}-ARs and α_{1D}-AR expressed alone, suggesting that this heterodimer may act as a high-efficacy complex. This is similar to previous findings on α_{1A}-/α_{1B}-AR heterodimerization in which NE responses were ~10-fold greater in HEK293 cells coexpressing α_{1A}- and α_{1B}-ARs (Israilova et al., 2004). In addition, a recent study using α_1-AR knockout mice found that α_{1D}-AR and α_{1D}-/α_{1B}-AR knockout mice had a significant decrease in mean arterial blood pressure, whereas α_{1B}-AR knockout mice did not, suggesting that α_{1D}-/α_{1B}-ARs may act cooperatively to regulate blood pressure (Hosoda et al., 2005). Additional evidence for a physiological role of α_{1B}-AR/α_{1D}-AR heterodimers was provided from functional studies of isolated mouse carotid arteries, in which the potency of phenylephrine was significantly decreased in α_{1D}-AR knockout mice yet unchanged in α_{1B}-AR knockout mice

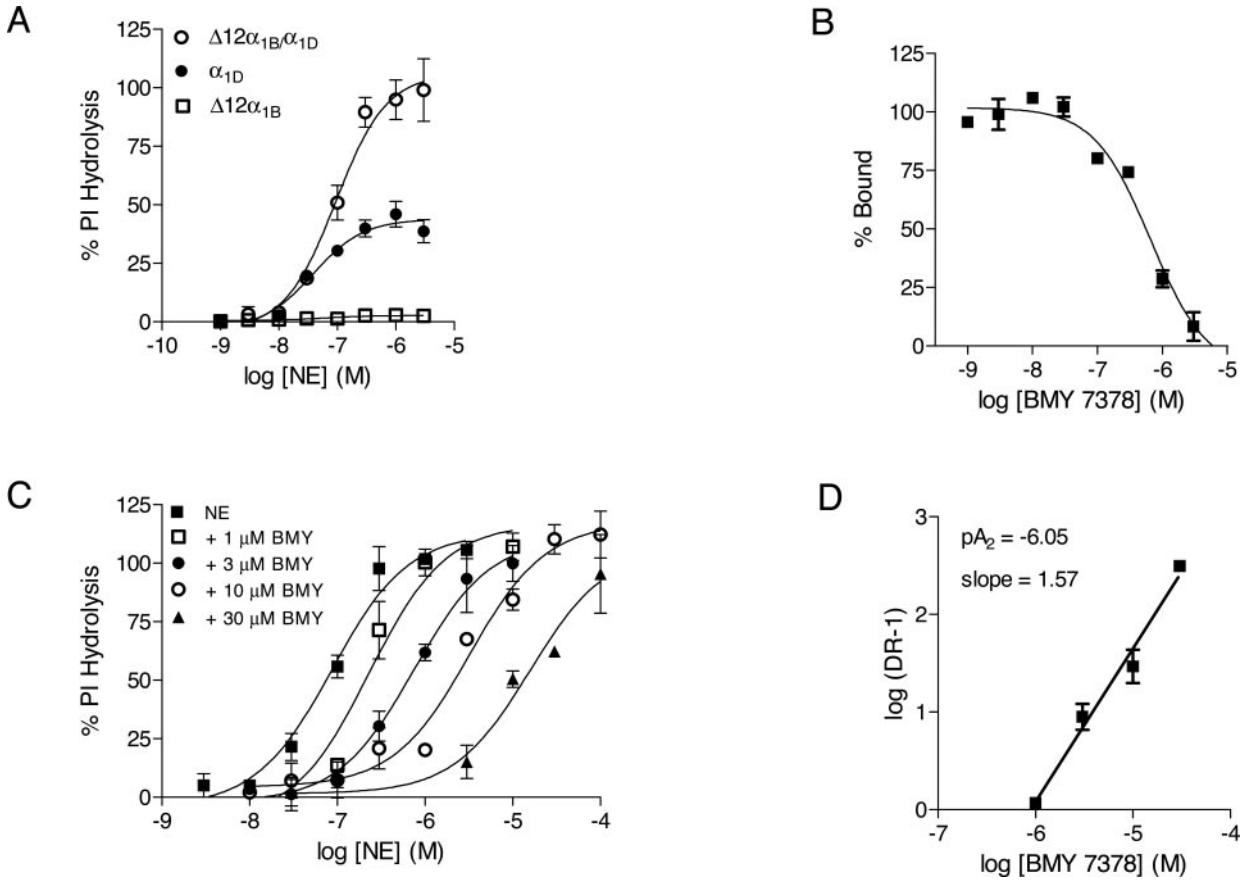

Fig. 7. α_{1B}- and α_{1D}-ARs form distinct components of a heterodimer signaling complex. A, coexpression of WT α_{1D}-ARs with functionally uncoupled $\Delta12\alpha_{1B}$-ARs increases NE maximal responses. HEK293 cells expressing WT α_{1D}-ARs alone (●), $\Delta12\alpha_{1B}$-ARs alone (□), or coexpressed $\Delta12\alpha_{1B}$-/α_{1D}-ARs (○) were incubated with [*myo*-^{3}H]inositol for 24 h. Cells were then stimulated with increasing concentrations of NE for 1 h and assayed for [^{3}H]InsP production as described in *Materials and Methods*. Data are expressed as the percentage of PI hydrolysis, with 100% stimulation equal to the level attained in cells coexpressing $\Delta12\alpha_{1B}$-/α_{1D}-ARs. Data are the means of three individual experiments performed in duplicate. B, ^{125}I-BE 2254 competition radioligand binding was used to determine BMY 7378 binding affinity in HEK293 cells coexpressing $\Delta12\alpha_{1B}$-/α_{1D}-ARs (■). Data are the means of three independent experiments performed in duplicate and are expressed as mean ± S.E.M. C, BMY 7378 inhibits NE functional responses in HEK293 cells coexpressing $\Delta12\alpha_{1B}$-/α_{1D}-ARs with α_{1B}-AR pharmacology. HEK293 cells stably expressing $\Delta12\alpha_{1B}$-/α_{1D}-ARs were stimulated with increasing concentrations of NE in the absence and presence of 1 μM (□), 3 μM (●), 10 μM (○), and 30 μM (▲) BMY 7378. Data are expressed as the percentage of PI hydrolysis with 100% stimulation equal to the NE maximum and are the mean ± S.E.M. of three individual experiments performed in duplicate. D, Schild plot of BMY 7378 inhibition of NE-stimulated [^{3}H]InsP production in HEK293 cells coexpressing $\Delta12\alpha_{1B}$-/α_{1D}-ARs.

(Deighan et al., 2005). These findings raise the possibility that specific heterodimers respond supermaximally to agonist stimulation. Previous studies have reported that the formation of receptor heterodimers results in altered receptor functional characteristics (Breit et al., 2004; Lee et al., 2004). Thus, another possibility is that α_{1B}-/α_{1D}-AR heterodimers are responsible for activating novel transcriptional activators or mitogenic pathways. Future studies are needed to test this hypothesis.

It is becoming increasingly clear that previously unexplained reports of altered pharmacological or functional characteristics of GPCRs may be explained by the formation of heterodimeric complexes. We have found that α_{1B}-/α_{1D}-AR heterodimers mask BMY 7378 high-affinity α_{1D}-AR binding sites, which may explain the inability of BMY 7378 to detect α_{1D}-AR binding sites in native tissues coexpressing α_{1B}- and α_{1D}-ARs. These results raise the possibility that the number of pharmacologically distinct receptor subtypes may be greater than would be predicted by the number of GPCR genes. If true, the use of heterologous systems expressing a single GPCR to screen for novel therapeutics may not accurately reflect the pharmacological complexity of a drug in vivo.

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