High-Affinity Interactions between Human α_{1A}-Adrenoceptor C-Terminal Splice Variants Produce Homo- and Heterodimers but Do Not Generate the α_{1L}-Adrenoceptor

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ABSTRACT

Using combinations of bioluminescence resonance energy transfer, time-resolved fluorescence resonance energy transfer and the functional complementation of pairs of inactive receptor-G protein fusion proteins, the human α_{1A,1}-adrenoceptor was shown to form homodimeric/oligomeric complexes when expressed in human embryonic kidney (HEK) 293 cells. Saturation bioluminescence resonance energy transfer studies indicated the α_{1A,1}-adrenoceptor homodimer interactions to be high affinity and some 75 times greater than interactions between the α_{1A,1}-adrenoceptor and the δ opioid peptide receptor. Only a fraction of the α_{1A,1}-adrenoceptors was at the plasma membrane of HEK293 cells at steady state. However, dimers of α_{1A,1}-adrenoceptors were also present in intracellular membranes, and the dimer status of those delivered to the cell surface was unaffected by the presence of agonist. Splice variation can generate at least three forms of the human α_{1A,1}-adrenoceptor with differences limited to the C-terminal tail. Each of the α_{1A,1}, α_{1A,2a}, and α_{1A,3a}-adrenoceptor splice variants formed homodimers/oligomers, and all combinations of these splice variants were able to generate heterodimeric/oligomeric interactions. Despite the coexpression of these splice variants in human tissues that possess the pharmacologically defined α_{1L}-adrenoceptor binding site, coexpression of any pair in HEK293 cells failed to generate ligand binding characteristic of the α_{1L}-adrenoceptor.

It is now generally accepted that G protein-coupled receptors (GPCRs) can exist as dimers or higher-order oligomers (Bouvier, 2001; Milligan, 2001; George et al., 2002). The ability to coimmunoprecipitate coexpressed but differentially epitope-tagged forms of a single GPCR was a key approach in early studies on GPCR homodimerization (Hebert et al., 1996; Cvejic and Devi, 1997; Salim et al., 2002). More recently, a range of techniques has been used to monitor GPCR interactions in intact cells (Angers et al., 2000; McVey et al., 2001; Carrillo et al., 2003; Stanasila et al., 2003). Resonance energy transfer techniques have been most actively used because the upper limit of distances commensurate with generating a resonance energy transfer signal is similar to the predicted dimensions of a GPCR dimer. In initial studies on dimerization of the β_{2}-adrenoceptor using bioluminescence resonance energy transfer (BRET), coexpression of forms of this receptor tagged at the C terminus with either Renilla reniformis luciferase (R-Luc) or enhanced yellow fluorescent protein produced data consistent with a significant degree of constitutive dimerization/oligomerization (Angers et al., 2000). Addition of an agonist for the receptor further increased the BRET signal, consistent with agonists increasing the fraction of the GPCR existing as a dimer. However, the exquisite dependence of resonance energy transfer signals with distance and orientation of energy donor and acceptor (Eidne et al., 2002) means that such results are also compatible with small movements within the dimer in response to agonist binding. Such an explanation has been discussed directly in studies of the effects of ligands on the

ABBREVIATIONS: GPCR, G protein-coupled receptor; HEK, human embryonic kidney; BRET, bioluminescence resonance energy transfer; GFP, green fluorescent protein; CQ, antiserum directed against the C-terminal decapeptide of Gq/G11; R-Luc, Renilla reniformis luciferase; Tr-FRET, time-resolved fluorescence resonance energy transfer; E, emission; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; BRET_{50}, half-maximal bioluminescence resonance energy transfer signal; DOP, δ opioid peptide; [^{35}S]GTP_{S}, 5’-O-[^{35}S]thio)triphosphate; QAPB, BODIPY-FL prazosin; ROQABP, red BODIPY-FL prazosin; XL665, alphapoycocyanin; TE, Tris/EDTA; CGP12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazole-2-one hydrochloride.
interactions between melatonin receptor subtypes (Ayoub et al., 2002). The effects of agonist ligands on GPCR homodimerization provides a complex literature, in which increases, decreases, and no alterations have been reported (Milligan, 2001; George et al., 2002).

GPCRs also have the potential to form heterodimeric/oligomeric complexes, and a series of studies have described alterations of ligand pharmacology after coexpression of pairs of GPCRs (Jordan and Devi, 1999; Rochelle et al., 2000). Such data suggest that GPCR heterodimerization might explain certain examples in which pharmacological observations are more complex than can be easily explained by ligand binding to single well-characterized GPCRs. This has been most actively studied after coexpression of pairs of opioid receptors (Devi, 2001). For example, coexpression of the \( \mu \)-opioid peptide and DOP receptors results in pharmacology of ligand binding and function that is not a simple mixture of those anticipated for the coexpressed but isolated receptors (Jordan and Devi, 1999; George et al., 2000; Martin and Prather, 2001). Despite a significant number of studies that support the concept of GPCR heterodimerization, quantification of the ability and selectivity of different GPCRs to form heterodimers remains relatively unexplored (Mercier et al., 2002; Ramsay et al., 2002).

Humans have three distinct genes that encode GPCRs with classic \( \alpha_1 \)-adrenoceptor pharmacology. This includes high-affinity binding of the antagonist prazosin (Piascik and Perez, 2001). Message encoding the \( \alpha_1A \)-adrenoceptor is predominant in prostate, and because it is suggested to mediate smooth muscle contraction in this tissue, it is a potential target for therapeutic intervention in benign prostatic hyperplasia (Pool and Kirby, 2001). However, the presence of an \( \alpha_1 \)-adrenoceptor–like binding site with low affinity for prazosin, named the \( \alpha_{1L} \)-adrenoceptor, has been shown in human prostate (Ford et al., 1997). Although there have been suggestions that the \( \alpha_{1L} \)-adrenoceptor represents a distinct functional state of the \( \alpha_1A \)-adrenoceptor (Ford et al., 1997), this remains unclear, and none of the classic human \( \alpha_1 \)-adrenoceptor sequences display the appropriate ligand binding characteristics when expressed separately in heterologous cell systems. There is also no orphan GPCR sequence in the human genome with substantial homology to the \( \alpha_1 \)-adrenoceptor grouping that is likely to correspond to this binding site (Fredriksson et al., 2003). It is thus possible that the \( \alpha_{1L} \)-adrenoceptor corresponds to a heterodimer containing the \( \alpha_1A \)-adrenoceptor. A number of both homo- and heterodimeric interactions between \( \alpha_1 \)-adrenoceptor subtypes have been reported (Vicentic et al., 2002; Carrillo et al., 2003; Stanasila et al., 2003; Uberti et al., 2003), but where examined, this does not alter the pharmacology of the ligand binding site. Further analysis of this possibility is encouraged, however, by the generation and expression of a number of splice variants of the \( \alpha_1A \)-adrenoceptor (Coge et al., 1999).

A number of the human \( \alpha_1A \)-adrenoceptor splice variants differ from the prototypic \( \alpha_1A \)-adrenoceptor only in sequences in the C-terminal intracellular tail region (Coge et al., 1999). The current study was thus designed to examine dimerization of the human \( \alpha_{1A,1} \)-adrenoceptor to provide means to analyze selectivity of GPCR heterodimerization, to assess interactions between C-terminal splice variants of the human \( \alpha_{1A} \)-adrenoceptor, and to explore whether coexpressed combinations of these produced \( \alpha_{1L} \)-adrenoceptor pharmacology.

## Materials and Methods

### Materials

Production and characterization of the anti-G\(_{\alpha}\)/G\(_{11}\) antiseraum CQ was described previously (Mitchell et al., 1993). Oligonucleotides were purchased from Thermohybid (Ulm, Germany). All materials for tissue culture were supplied by Invitrogen (Paisley, Strathclyde, UK). (\(^{3}H\))Prazosin (80 Ci/mmol) and (\(^{35}S\)GTP\(\gamma\)S (1250 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA). (\(^{3}H\))Diprenorphine (50 Ci/mmol) was from Amersham Biosciences Inc. (Piscataway, NJ). Reagents for time-resolved fluorescence energy transfer (Tr-FRET) were from PerkinElmer Life and Analytical Sciences. All reagents for BRET\(^2\) were from PerkinElmer Life and Analytical Sciences. BODIPY-FL prazosin (QAPB) (Daly et al., 1998) and red QAPB (RQAPB) were from Molecular Probes (Eugene, OR). Receptor ligands were purchased from Sigma/RBI (Gillingham, Kent, UK). All other chemicals were from Sigma Chemical (Poole, Dorset, UK) or Fisons (Loughborough, United Kingdom) and were of the highest grade available.

### Construction of Receptor Plasmids

Production and subcloning of Dop–R-Luc was performed as described previously (McVey et al., 2001), as was subcloning of Dop-green fluorescent protein\(^2\) (GFP\(^2\)) (Ramsay et al., 2002). Production and subcloning of \( \alpha_{1A,1} \)-adrenoceptor–R-Luc involved PCR of the human \( \alpha_{1A,1} \)-adrenoceptor sequence. Using the amino-terminal primer 5'-AAA AGG TAC CAT GGT GTT TTC CTC CGG AAA TGG TTG TTC-3' and KpnI restriction sequence was introduced upstream of the coding sequence. Using the carboxy-terminal primer 5'-AAA AGG GCC GGC GAC TTC TCT CCC GTT TTC CTC ACT GAG GGG-3', the receptor stop codon was removed, and a NotI restriction enzyme site introduced downstream of the receptor coding sequence. Similarly R-Luc was PCR-amplified using the amino-terminal primer 5'-AAAG CGG CCG CTA CTT CGA AAG TTT ATG-3' to introduce a NotI restriction sequence upstream of the coding sequence. The carboxy-terminal primer 5'-GGG TCT AGA TTA TTG TTC ATT TT-3' was used to introduce an XbaI restriction enzyme site immediately downstream from the stop codon. The fragments thus generated were then directly ligated into the expression vector pcDNA3.

Production and subcloning of \( \alpha_{1A,1} \)-adrenoceptor-GFP\(^2\) involved PCR of the human \( \alpha_{1A,1} \)-adrenoceptor sequence using amino-terminal primer 5'-AAA AGG TAC CAT GTT GGT TTC CTC CGG AAA TGG TTG-3' to introduce a KpnI restriction sequence upstream of the coding sequence. The carboxy-terminal primer 5'-AAA AGG TAC CAT GGT GGT TTC CTC CGG AAA TGG TTG-3' was used to introduce the FLAG or c-myc sequences, respectively, as well as a BamHI restriction enzyme site downstream of the receptor coding sequence. The resulting PCR fragments were then ligated into pGFP2 N2 vector (PerkinElmer).

For Tr-FRET studies, c-myc (EQKLISEEDL) or FLAG (DYKD-DSDK) epitope tags were introduced immediately upstream of each of the human \( \alpha_{1A,1} \), \( \alpha_{1A,1,2a} \), and \( \alpha_{1A,1,3b} \)-adrenoceptor splice variants. The amino-terminal primers 5'-AAA AGG TAC CAT GGT GGT TTC CTC CGG AAA TGG TTG-3' or 5'-AAA AGG TAC CAT GGA ACA AAA ACT TAT TTC TGA AGA AGA TCT TTC GGG AAA TGG TTC C-3' were used to introduce the FLAG or c-myc sequences, respectively, as well as a KpnI site, upstream of the receptor. Depending on the splice variant used as template, the following carboxy-terminal primers were used: \( \alpha_{1A,1} \)-adrenoceptor, 5'-AAA AGG ATC CCT AGA CTT CCT CCC GGT TCT CAC TGA GGG-3' incorporating a BamHI site downstream of the coding sequence; \( \alpha_{1A,1,2a} \)-adrenoceptor, 5'-GGG CTC TAG ATC ATG AGG TCA AGA CAT CG-3' incorporating an XbaI site downstream of the coding sequence; and \( \alpha_{1A,1,3b} \)-adrenoceptor, 5'-GGT CTC TAG ATC ATG AGG TCA TG GGG TTC G-3' incorporating an XbaI site downstream of the coding sequence. All PCR fragments were subsequently cloned into pcDNA3.
Construction of the wild-type α1A-1-adrenoceptor–Gq11 fusion protein required PCR amplification of both α1A-1-adrenoceptor and Gq11. PCR of the α1A-1-adrenoceptor used an amino-terminal primer 5'-TTA GGC AAC TTT GCC ACC ATG TAG CAA AAG CTT ATC TTT GAA GAG GAC TTT GTG TGT TCT TCG GGA AAT GC-3' to introduce a c-myc epitope tag immediately upstream of the receptor coding sequence as well as a HindIII restriction site. Using the carboxyl-terminal primer 5'-AGC ATT TCA AGC GCC CGC TGA GGT CAA GAC ATC AGC ATC-3’, the receptor stop codon was removed, and a NolI restriction enzyme site introduced downstream of the receptor coding sequence. Gq11 was PCR-amplified using the amino-terminal primer 5’-A AGC ATT TCA GGG GCC GCA ACT CTG GAG TCC ATG GAT G-3’. This introduced a NolI restriction sequence upstream of the coding sequence. The carboxyl-terminal primer 5’-ACA GTT CTC GAG TCA CAG CAG GTT GTC CTC C-3’ was used to introduce an XbaI restriction enzyme site immediately downstream of the stop codon. The fragments thus generated were then ligated into the expression vector pcDNA3. Construction of the adrenoceptor in the -GGT TGG GTA GCG GTC CGG GTA GCT generated mini-fragments along with the primers 5’/H11032 ATG ATG G-3’/H11032 A AGC ATT TCA GCG GCC GCA ACT CTG GAG TCC ATG ATG G-3’/H11032 and the carboxyl-terminal primer 5’-AGC ATT TCA GGG GCC GCA ACT CTG GAG TCC ATG GAT G-3’. This introduced a NolI restriction sequence downstream of the coding sequence. The carboxyl-terminal primer 5’-ACA GTT CTC GAG TCA CAG CAG GTT GTC CTC C-3’ and the carboxyl-terminal primer 5’-AGC ATT TCA GGG GCC GCA ACT CTG GAG TCC ATG GAT G-3’, which removed the stop codon and introduced a NolI restriction sequence downstream from the receptor coding sequence. A further round of PCR was performed using the above-generated mini-fragments along with the primers 5’-TTA GGA ATT CGC CAC CAT GGA CTA CAA GGA CGA CGA TGA CAA GGT TTT TCT CTC GGG AAA TCG-3’-3’ and the carboxyl-terminal primer 5’-GTG AGC TAC CCG GAC CGC TAC CCA ACC-3’, which produced the Leu132Asp substitution; and 2) using the amino-terminal primer 5’-GGT TGG TGG ATC GGC CGC TGA GCT GTG CTC GAT CAC-3’, containing the Leu132Asp substitution and the carboxyl-terminal primer 5’-AGC ATT TCA GGG GCC GCA ACT CTG GAG TCC ATG GAT G-3’, which removed the stop codon and introduced a NolI restriction sequence downstream from the receptor coding sequence. A further round of PCR was performed using the above-generated mini-fragments along with the primers 5’-TTA GGA ATT CGC CAC CAT GGA CTA CAA GGA CGA CGA TGA CAA GGT TTT TCT CTC GGG AAA TCG-3’-3’ and the carboxyl-terminal primer 5’-GTG AGC TAC CCG GAC CGC TAC CCA ACC-3’, which produced the Leu132Asp substitution; and 2) using the amino-terminal primer 5’-GGT TGG TGG ATC GGC CGC TGA GCT GTG CTC GAT CAC-3’, containing the Leu132Asp substitution and the carboxyl-terminal primer 5’-AGC ATT TCA GGG GCC GCA ACT CTG GAG TCC ATG GAT G-3’, which removed the stop codon and introduced a NolI restriction sequence downstream from the receptor coding sequence. The homogenate was centrifuged at 500,000 g for 30 min, and the supernatant was resuspended in ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, and 10 mM MgCl₂, pH 7.4) and lysed with two 10-s sonication bursts in a Polytron homogenizer (Kinematica, Basel, Switzerland). The homogenate was centrifuged at 500,000 g for 30 min, and the supernatant was resuspended in TB buffer and stored at −80°C until use. [35S]GTPγS binding studies were performed on membrane preparations from HEK293 cells. Membrane protein (10 μg) was added to tubes containing 50 mM Tris-HCl, 1 mM EDTA, and 10 mM MgCl₂, pH 7.4, and [35S]GTPγS (variable concentrations) in the absence or presence of 10 μM unlabeled prazosin to define non-specific binding at 30°C for 30 min. [3H]Diprenorphine binding studies were also performed on membrane preparations from HEK293 cells. Membrane protein (10 μg) was added to an assay mix containing 50 mM Tris-HCl, 1 mM EDTA, and 10 mM MgCl₂, pH 7.4, and [3H]diprenorphine (variable concentrations) in the absence or presence of 100 μM unlabeled naloxone as a competitor at 30°C for 30 min. In all cases, bound ligand was separated from free by vacuum filtration through GF/B filters. Filters were washed three times with ice-cold TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.4), and the quantity of bound ligand was then determined by liquid scintillation spectrometry.
membranes to an assay buffer (20 mM HEPES, pH 7.4, 3 mM MgCl₂, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 50 nCi of [³²S]GTPγS) containing the indicated concentrations of receptor ligands. Nonspecific binding was determined under the same conditions but in the presence of 100 μM GTPγS. Reactions were incubated for 15 min at 30°C and were terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM MgCl₂, and 100 mM NaCl. The samples were centrifuged at 16,000 g for 15 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA and 1.25% Nonidet P-40) plus 0.2% SDS. Samples were preclariﬁed with Pan sorbin (Calbiochem, San Diego, CA) followed by immunoprecipitation with CQ antiserum (Mitchell et al., 1993). Finally, the immunocomplexes were washed twice with solubilization buffer, and bound [³²S]GTPγS was measured by liquid scintillation spectrometry (Liu et al., 2002).

Duo- Density Gradient Preparation. Dishes (10 × 10 cm) were cotransfected with c-myc α₁A₁-adrenoceptor and FLA Gα₁s/1₆- adrenoceptor, or, as a negative control, 5 × 10 cm dishes were singly transfected with c-myc α₁A₁-adrenoceptor or FLA Gα₁s/1₆-adrenoceptor. Forty-eight hours later, these were harvested in PBS. At this point, the cell populations singly expressing c-myc α₁A₁-adrenoceptor and FLA Gα₁s/1₆-adrenoceptor were mixed. Cells were pelleted by centrifugation at 15,000 rpm in a swing-bucket rotor. The cell pellets were resuspended and then homogenized for 7 min in 2 ml of a buffer containing 0.25 M sucrose, 50 mM Tris-HCl, 3.0 mM MgCl₂, and 1 mM EDTA plus a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The homogenate was transferred to a centrifuge tube and overlaid with 2 ml of 80% sucrose solution. This was then further overlaid with 1.5-mL volumes of 35%, 30%, 25%, 20%, and 15% solutions of sucrose in 20 mM Tris and 1 mM EDTA buffer, pH 7.4. Homogenates were then centrifuged at 39,000 rpm for 24 h in a Beckman SW40 swing-out rotor (Beckman Coulter, Inc., Fullerton, CA). After centrifugation, the samples were separated into 12 fractions of equal volume with the earlier fractions corresponding to lower densities. These were diluted 1 in 2 with distilled H₂O and then centrifuged at 240,000 g for 30 min to pellet the membrane fractions. The resultant pellets were then incubated with both anti-c-myc-Eu³⁺ and anti-FLAG XLE665 antibodies together at final concentrations of 5 and 15 nM, respectively, in buffer containing 50% newborn calf serum/50% PBS (200 μl) for 3 h at room temperature. After incubation, the membranes were diluted with 1 ml of PBS and centrifuged at 240,000 g for 30 min. Pellets were washed and then resuspended, as described above. Finally the pellets were resuspended in 200 μl of PBS and assayed according to the Tr-FRET protocol described previously (McVey et al., 2001). The background ratio of E₆₀₀/E₆₁₅ obtained from the mixed cell samples was subtracted from the ratio E₆₀₀/E₆₁₅ obtained from coexpressed constructs to obtain a FRET reading minus background. Analysis of the distribution in such gradients of protein, the β₁-adrenoceptor that is expressed endogenously by HEK293 cells, and the plasma membrane markers adenylyl cyclase and the ouabain-sensitive Na⁺/K⁺ ATPase was conducted as described previously (Bourouva et al., 2003).

Confocal Laser Scanning Microscopy. Cells expressing different fluorophores were imaged using a Zeiss 5 PASCAL laser scanning confocal microscope equipped with a 63× oil-immersion Plan Apochromat objective lens (numerical aperture = 1.4) (Carl Zeiss Inc., Thornwood, NY). The following laser lines were used for excitation: 488 nm for GFP², and 543 nm for a red variant (RQAPB) of the fluorescent α₁-adrenoceptor antagonist ligand QAPB. The following Zeiss filter sets were used to detect the fluorescence of each fluorophore: BP505-530 for GFP², and LP570 for RQAPB. Recorded 12-bit images were exported into MetaMorph imaging software (version 6.1.3; Universal Imaging Corporation, Downing, PA) to create overlay images.

Live cells were used for all experiments, and cells were maintained in Dulbecco’s phosphate-buffered saline.

Dual GFP² and RQAPB Confocal Imaging. HEK293 cells were plated onto sterile round glass coverslips (22 mm), and after a 24-h growth period were transiently transfected with cDNA encoding a GFP²-tagged version of the human α₁A₁-adrenoceptor. Transfected cells were cultured overnight, and the growth medium then was removed and replaced with fresh PBS. The cells were then pre-equilibrated at 37°C with fresh PBS containing 10 nM RQAPB for 75 min. The RQAPB-treated cells were mounted onto an imaging chamber, and using the appropriate laser lines, sequential images were acquired to determine the total GFP² and RQAPB fluorescence emission intensity associated with each transfected cell.

Dual Hoechst and QAPB Epifluorescence Imaging. Cells transiently transfected to express the human α₁A₁-adrenoceptor were rinsed several times in PBS and then incubated at 37°C with PBS supplemented with 10 nM concentrations of the green fluorescent α₁-adrenoceptor antagonist ligand QAPB for 70 min. Cell nuclei were subsequently stained by incubating the cells for 5 min at 37°C with fresh PBS containing the nuclear DNA-binding dye Hoechst 33342 (10 μg/ml) plus QAPB (10 nM). Cells were then washed several times with PBS supplemented with QAPB only before image acquisition. Hoechst and QAPB fluorescence emission was detected using a DIA phot inverted microscope equipped with a 40× oil-immersion Fluor objective lens (numerical aperture = 1.3) (Nikon, Melville, NY). A monochromator (Optoscan; Cairn Research, Faversham, Kent, UK) was used for the sequential excitation of Hoechst (350 nM) and QAPB (490 nm). Hoechst and QAPB fluorescence emission was detected by a cooled digital charge-coupled device camera (Cool Snap-HQ; Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (version 4.6.9; Universal Imaging Corporation) was used for control of the monochromator and the charge-coupled device camera and for processing of the cell image data. Sequential images (no binning) were collected at 15-s intervals, and exposure to excitation light was 40 ms/image.

Data Analysis. All experiments were performed on at least three independent occasions. Where appropriate, data are presented as means ± S.E.M.

Results

BRET has been used extensively to monitor both homodimeric/oligomeric interactions betweenGPCRs in living cells (Angers et al., 2000; McVey et al., 2001; Eidne et al., 2002). Energy transfer between polypeptides tagged with either R-Luc or enhanced yellow fluorescent protein has been the most popular form of BRET. The improved signal to background that is achieved when using DeepBlueC as substrate for the luciferase when GFP² is the energy acceptor, however, has recently resulted in significant use of BRET² (Mercier et al., 2002; Ramsay et al., 2002). Forms of the human α₁A₁-adrenoceptor tagged at the C terminus with either R-Luc or GFP² were generated and expressed transiently in HEK293 cells. The binding affinity of the α₁-adrenoceptor antagonist/inverse agonist [³H]prazosin to these constructs in cell membrane preparations was not significantly different (Kᵦ for α₁A₁-adrenoceptor–R-Luc = 0.71 ± 0.18 nM; Kᵦ for α₁A₁-adrenoceptor–GFP² = 0.95 ± 0.10 nM) but was some 3-fold lower than the unmodified human α₁A₁-adrenoceptor (Kᵦ = 0.28 ± 0.04 nM).

Coexpression of α₁A₁-adrenoceptor–R-Luc and α₁A₁-adrenoceptor–GFP² in HEK293 cells followed by the addition of DeepBlueC resulted in a BRET² signal consistent with these two forms of the receptor forming a constitutive complex (Fig. 1). Addition of adrenaline (10 μM) did not modify the BRET² signal (Fig. 1), indicating that agonist binding did not alter
whether the DOP receptor or the H9251 expression of differing amounts of absolute luminescence and fluorescence signals after the ex-GPCRs to interact (Mercier et al., 2002). We monitored the not inherently informative on the relative propensity of et al., 2002). Thus, the absolute levels of BRET signals are sensitive to small differences in the distance between, and the orientations of, the energy donor and acceptor species (Eidne 2002). Resonance energy transfer signals are exquisitely sen-

wished to explore if these represented high affinity interac-

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sitive to small differences in the distance between, and the
gorientation of, the energy donor and acceptor species (Eidne 2002). Thus, the absolute levels of BRET signals are not inherently informative on the relative propensity of GPCRs to interact (Mercier et al., 2002). We monitored the absolute luminescence and fluorescence signals after the expression of differing amounts of α1A,1-adrenoceptor–R-Luc, DOP–R-Luc, α1A,1-adrenoceptor–GFP2, or DOP–GFP2 and correlated these with expression levels monitored by saturation binding studies using the antagonists [3H]prazosin (α1A,1-adrenoceptor) or [3H]diprenorphine (DOP receptor). In each case, receptor expression levels were linearly correlated with signal (Fig. 2). However, both luminescence (Fig. 2A) and fluorescence (Fig. 2B) signals were significantly greater per femtomole of the DOP receptor constructs than for the α1A,1-adrenoceptor constructs. Although unexpected, similar observations have been noted previously in direct comparisons between equivalently tagged forms of the β2- and β1- adrenoceptors (Mercier et al., 2002). Saturation BRET2 experiments (Mercier et al., 2002) were then performed. In these, the ratio of the energy acceptor GPCR-GFP2 to energy donor GPCR–R-Luc was varied over a substantial range. With an increasing ratio of acceptor to donor, it is expected that a maximal BRET signal will be reached when all donor molecules interact with an acceptor. Expression ratios of acceptor to donor were calculated by converting fluorescence and luminescence data into receptor equivalents (Fig. 2). For the α1A,1-adrenoceptor pair, BRET2 signals increased as a hyperbolic function with increasing acceptor-to-donor ratio, reaching an asymptote of 0.38 ± 0.011 (n = 3) (Fig. 3A). Half-maximal BRET signal (BRET50) was achieved at an estimated α1A,1-adrenoceptor–GFP2/α1A,1-adrenoceptor–R-

Fig. 1. Hom- and heterointeractions of α1A,1-adrenoceptor and DOP-opioid receptors. BRET2-competent combinations of the human α1A,1-adrenoceptor and the DOP opioid receptor were expressed transiently in HEK293 cells. Constitutive interactions (□) and the effects on this of agonist treatment (all other bar fills) were measured after the addition of DeepBlueC.
Luc ratio of 4.56 ± 0.70, consistent with a high-affinity interaction. Equivalent studies using the DOP receptor BRET5 pair also generated a hyperbolic function with asymptote of 1.14 ± 0.071 (n = 3) (Fig. 3B) and a measured BRET50 acceptor-donor ratio of 9.8 ± 3.1. The ability to express substantially higher levels of the α1A-1-adrenoceptor constructs than the DOP receptor constructs (Fig. 2) allowed the effectiveness of heterointeractions to be measured by using DOP–R-Luc as energy donor and α1A-1-adrenoceptor–GFP2 as energy acceptor. In saturation BRET5 studies, the energy transfer signal between DOP–R-Luc and α1A-1-adrenoceptor–GFP2 also described a hyperbolic function with, in this case, an asymptote of 0.21 ± 0.02 (n = 3) (Fig. 3C). However, in this case, the BRET50 energy acceptor-donor ratio was 356 ± 110 (n = 3), consistent with an affinity of interaction between these two GPCRs to form a heterocomplex that was much lower than for either of the two homo-oligomers (Fig. 3).

As an alternative strategy to monitor human α1A-1-adrenoceptor dimerization, we used pairs of nonfunctional but potentially complementary GPCR-G protein α-subunit fusion proteins (Carrillo et al., 2003). The α subunit of the Ca2+–mobilizing G protein G11 was linked in-frame to the C-terminal tail of the α1A-1-adrenoceptor. This construct was expressed in HEK293 cells, and membranes were prepared. The binding affinity of [3H]prazosin to this construct was similar (0.29 ± 0.02 nM, n = 3) to the wild-type α1A-1-adrenoceptor. Membrane amounts containing 25 fmol of [3H]prazosin binding sites were used in [35S]GTPyS binding assays performed in the absence or presence of the α1-adrenoceptor agonist phenylephrine (100 μM). At the termination of the assay, samples were immunoprecipitated with an antiserum, CQ (Mitchell et al., 1993), directed against the C-terminal decapeptide of G11 and counted (Fig. 4). Little binding of [35S]GTPyS was observed in the absence of agonist, indicating, as shown previously for the hamster α1B-adrenoceptor (Carrillo et al., 2002), limited constitutive capacity of the human α1A-1-adrenoceptor to activate G11. The presence of phenylephrine, however, resulted in a large stimulation of [35S]GTPyS binding in the immunoprecipitate (Fig. 4). Gly208Ala G11 is unable to bind GTP or its analogs (Carrillo et al., 2002, 2003). When Gly208Ala G11 was linked in-frame to the α1A-1-adrenoceptor and this construct expressed in HEK293 cells, both agonist and antagonist ligands at the receptor bound with affinities similar to those at the wild-type fusion protein (Table 1). In contrast, when membranes expressing the same number of [3H]prazosin binding sites were used in [35S]GTPyS binding studies as for the wild-type fusion protein, phenylephrine produced only a very small increase in [35S]GTPyS binding (Fig. 4). Mutation of the hydrophobic Leu132 residue in the second intracellular loop of the α1A-1-adrenoceptor to aspartic acid also resulted in the loss of phenylephrine stimulation of [35S]GTPyS binding when this form of the receptor was coupled to wild-type G11 (Fig. 4). Again, this did not result from significant alterations in the binding of agonist or antagonist ligands (Table 1). However, when the two essentially nonfunctional α1A-1-adrenoceptor–G11 fusion proteins were coexpressed in HEK293 cells and membranes containing 25 fmol of [3H]prazosin binding sites used in [35S]GTPyS binding assays, phenylephrine-induced binding of the nucleotide was reconstituted (Fig. 4). Agonist stimulation of [35S]GTPyS binding was not produced when membrane preparations, each expressing one
of the inactive but potentially complementary fusion proteins, were combined before assay (Fig. 4), confirming the requirement for physical proximity to produce dimerization and reconstitution of function (Carrillo et al., 2003).

Neither BRET nor the fusion protein complementation approach can provide significant information on the cellular location of α1A-1-adrenoceptor dimers, and indeed, a number of studies have shown significant populations of intracellular α1-adrenoceptor subtypes (Hirasawa et al., 1997; Daly et al., 1998). When expressed in HEK293 cells, confocal microscopy indicated that a significant amount of the human α1A-1-adrenoceptor–GFP2 construct was not located at the plasma membrane. Although excluded from the nucleus, internal membranes displayed strong GFP2 fluorescence (Fig. 5A). Addition of a red variant (RQAPB) of the fluorescent α1-adrenoceptor antagonist QAPB (10 nM) (Hirasawa et al., 1997; Daly et al., 1998) to these cells resulted in an equivalent pattern of staining (Fig. 5B), confirming that the green fluorescence did indeed reflect the distribution of the expressed GFP2-tagged α1A-1-adrenoceptor. The significant intracellular accumulation of the expressed α1A-1-adrenoceptor–GFP2 construct did not result simply from the addition of GFP2. When HEK293 cells were transfected to express the isolated human α1/2/H9251 fluorescence did indeed reflect the distribution of the expressed α1A-1-adrenoceptor–GFP2 construct and counted. Data represent means ± S.E.M. from three independent experiments.

TABLE 1

<table>
<thead>
<tr>
<th>Construct</th>
<th>[3H]Prazosin Kd (nM)</th>
<th>Phenylephrine Kq (μM)</th>
<th>Adrenaline Ki (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTα1A-1, Ga11</td>
<td>0.29 ± 0.02</td>
<td>15.8 ± 9.9</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>L132D D11α1A, Ga11</td>
<td>0.24 ± 0.03</td>
<td>30.8 ± 12.0</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>α1A-1, G208AGa11</td>
<td>0.27 ± 0.04</td>
<td>28.3 ± 12.7</td>
<td>6.3 ± 2.1</td>
</tr>
</tbody>
</table>

WT, wild type.

To examine dimerization/oligomerization specifically of the population of the α1A-1-adrenoceptor that did reach the cell surface, we used Tr-FRET (McVey et al., 2001; Carrillo et al., 2003). Forms of the α1A-1-adrenoceptor were modified at the extreme N terminus to encode either FLAG or c-myc epitope tag sequences and coexpressed in HEK293 cells. Coaddition of Eu3+-labeled anti–c-myc antibodies as energy donor and XL665-labeled anti-FLAG antibodies as potential energy acceptors to the intact, transfected cells resulted in a positive energy transfer signal monitored as emission of light at 665 nM when the cells were illuminated at 330 nM to produce long-lived fluorescence from Eu33+ (Fig. 6). Energy transfer was not produced when only the Eu3+-labeled anti–c-myc antibodies were added or if HEK293 cells separately expressing the FLAG or c-myc tagged forms of the α1A-1-adrenoceptor were mixed before the addition of the antibodies (Fig. 6). As noted in the BRET2 studies, the addition of adrenaline (10 μM) did not alter the energy transfer signal (Fig. 6). As with the BRET2 studies, it was important to use a GPCR that has only limited interactions with the α1A-1-adrenoceptor. We have shown previously only weak interactions at the cell surface between the α1B-adrenoceptor and the histamine H1 receptor (Carrillo et al., 2003). Coexpression of N-terminally tagged forms of the α1A-1-adrenoceptor and the histamine H1 receptor resulted in a very limited Tr-FRET signal upon the addition of the combination of Eu33+-labeled anti–c-myc and XL665-labeled anti-FLAG antibodies (Fig. 6).

To determine whether the α1A-1-adrenoceptors located in-
tracellular membranes were also present as dimers/oligomers, HEK293 cells transfected to express a combination of FLAG- and c-myc-tagged forms of the \( \alpha_{1A-1} \)-adrenoceptor were homogenized, and the samples were centrifuged on sucrose density gradients. Fractions were then recovered, and both Eu\(^{3+} \)-labeled anti-c-myc antibodies and XL665-labeled anti-FLAG antibodies added. Energy transfer signals consistent with dimers/oligomers were detected in two distinct regions of the gradient, a "light vesicle" fraction shown previously to be enriched for endoplasmic reticulum/Golgi markers (Drmota et al., 1998) as well as in more dense fractions (Fig. 7). \[^{3}H\]Prazosin binding studies confirmed the bipolar distribution of \( \alpha_{1A-1} \)-adrenoceptor binding sites in such gradients (Fig. 7) and that the intensity of dimer/oligomer Tr-FRET signals in individual gradient fractions were similar to the distribution of the \( \alpha_{1A-1} \)-adrenoceptor. The denser gradient fractions containing the \( \alpha_{1A-1} \)-adrenoceptor were also enriched with both the ouabain-sensitive Na\(^+\)/K\(^+\) ATPase (Fig. 7) and adenyl cyclase activity (data not shown). Both of these are recognized markers of the plasma membrane (Bourova et al., 2003). HEK293 cells also endogenously express low levels of the \( \beta_2 \)-adrenoceptor. \[^{3}H\]CGP12177 binding studies demonstrated a single peak comigrating with the plasma membrane markers (Fig. 7).

A number of splice variants of the human \( \alpha_{1A} \)-adrenoceptor have been reported to occur (Coge et al., 1999). \( \alpha_{1A-1} \), \( \alpha_{1A-2a} \), and \( \alpha_{1A-3a} \) are forms of this GPCR that differ only in the sequence and length of C-terminal tail, with each containing seven transmembrane-spanning elements (Coge et al., 1999). These variants are coexpressed in prostate as well as other tissues (Coge et al., 1999). Because \( \alpha_{1L} \) pharmacology, an \( \alpha_{1} \)-adrenoceptor binding site with significantly lower affinity for prazosin than other \( \alpha_{1} \)-adrenoceptor sites, is present in prostate and has been indicated as a potentially useful target for therapeutic intervention in benign prostatic hypertrophy, we explored potential interactions between these splice variants. The splice variation resulting in the \( \alpha_{1A-2a} \) and \( \alpha_{1A-3a} \) forms alters the length and sequence of the C-terminal tail from that of the \( \alpha_{1A-1} \) receptor. We thus used Tr-FRET rather than BRET because the reporters are attached to the N terminus of the GPCRs in Tr-FRET, and this region is identical in the various splice variants, and because we wished to specifically monitor the profile of GPCR pairs that reached the cell surface. All of the homodimeric pairs (FLAG–\( \alpha_{1A-1} \)-adrenoceptor + c-myc–\( \alpha_{1A-1} \)-adrenoceptor, FLAG–\( \alpha_{1A-2a} \)-adrenoceptor + c-myc–\( \alpha_{1A-2a} \)-adrenoceptor, and FLAG–\( \alpha_{1A-3a} \)-adrenoceptor + c-myc–\( \alpha_{1A-3a} \)-adrenoceptor) generated Tr-FRET energy transfer signals that were not significantly different between the various pairings (Fig. 8). These were not observed when only Eu\(^{3+} \)-labeled anti-c-myc antibodies were added (Fig. 8). In each case, the addition of adrenaline (10 \( \mu \)M) did not alter the energy transfer signal (Fig. 8). Equally, coexpression of the c-myc–\( \alpha_{1A-1} \)-adrenoceptor with either FLAG–\( \alpha_{1A-2a} \)-adrenoceptor or FLAG–\( \alpha_{1A-3a} \)-adrenoceptor resulted in production of a similar level of energy transfer signal, consistent with constitutive heterodimerization (Fig. 8). Again, the addition of adrenaline (10 \( \mu \)M) did not alter the energy transfer signals (Fig. 8). Equally, coexpression of FLAG–\( \alpha_{1A-2a} \)-adrenoceptor and c-myc–\( \alpha_{1A-3a} \)-adrenoceptor resulted in Tr-FRET signals consistent with constitutive heterodimerization (Fig. 8). Whether measured in intact cells (data not shown) or in cell membranes (Table 2), the binding affinity of \[^{3}H\]prazosin for the individually expressed \( \alpha_{1A-1} \), \( \alpha_{1A-2a} \), and \( \alpha_{1A-3a} \) isoforms was not substantially different. Coexpression of the various splice variants did not generate a low-affinity binding site for \[^{3}H\]prazosin (Table 2) or result in a significant alteration in binding characteristics for other ligands useful in defining the \( \alpha_{1L} \)-adrenoceptor binding site (data not shown).
Discussion

Many GPCRs are capable of existing in both homo- and heterodimeric complexes (Bouvier, 2001; Milligan, 2001). There is also an emerging literature that GPCR heterodimers may display distinct pharmacology from the corresponding pairs of homodimers (Devi, 2001; George et al., 2002). Such observations demand understanding of the basis of GPCR dimerization and its selectivity. Data are beginning to emerge on the elements and interfaces of GPCRs that contribute to dimerization. Some early studies suggested an important role for the C or N terminus in certain family A GPCRs. However, most recent studies on family A GPCRs have focused on the transmembrane helices. Peptide competition studies at the β₂-adrenoceptor (Hebert et al., 1996) and the BLT1 leukotriene B4 receptor (Baneres and Parello, 2003) are consistent with a key role for transmembrane helix VI. By contrast, cysteine cross-linking studies indicate the importance of transmembrane helix IV in the D2 dopamine receptor (Guo et al., 2003), and atomic force microscopy stud-

Fig. 6. Detection of cell surface α₁A₁-adrenoceptor dimers using Tr-FRET. A, HEK293 cells were transfected to coexpress or separately express N-terminally FLAG- and c-myc–tagged forms of the α₁A₁-adrenoceptor. Cells were harvested, and either Eu³⁺-labeled anti–c-myc antibodies were added alone or in combination with XL665-labeled anti-FLAG antibodies. Tr-FRET signals were measured in the absence or presence of adrenaline (10 μM). B, the c-myc–α₁A₁-adrenoceptor was coexpressed with FLAG-tagged forms of either the α₁A₁-adrenoceptor or the histamine H1 receptor. In parallel, cells were transfected to express only c-myc–α₁A₁-adrenoceptor, the FLAG–α₁A₁-adrenoceptor, or the FLAG-histamine H1 receptor. c-myc and FLAG receptor-expressing cells were then mixed. Tr-FRET signals were measured after addition of both Eu³⁺-labeled anti–c-myc antibodies and XL665-labeled anti-FLAG antibodies.

Fig. 7. The α₁A₁-adrenoceptor exists as dimers/oligomers in multiple cellular locations. HEK293 cells were transfected to coexpress N-terminally FLAG- and c-myc–tagged forms of the α₁A₁-adrenoceptor. Cells were harvested and homogenized. Aliquots of the homogenate were applied to sucrose density gradients and centrifuged as described under Materials and Methods. Fractions of the gradient were taken, and membranes were recovered by dilution of the sucrose, centrifugation, and washing. A, Eu³⁺-labeled anti-c-myc antibodies and XL665-labeled anti-FLAG antibodies were added to membranes derived from equal volumes of individual sucrose density gradient fractions, and Tr-FRET was measured. Parallel studies measured [³H]prazosin binding (B), protein (C), [³H]CGP12177 binding (D), and the binding of [³H]ouabain (E). Lower numbers are lower density fractions from the gradient.
ies on rhodopsin dimers in situ support roles for both transmembrane helices IV and V (Liang et al., 2003). Other approaches have suggested important roles for transmembrane helix I in the α1A-adrenoceptor (Carrillo et al., 2003; Stanasila et al., 2003) and the yeast pheromone receptor (Overton and Blumer, 2002). Studies of heterodimer selectivity require careful controls because the hydrophobic transmembrane core of GPCRs is likely to provide mutual affinity, particularly when removed from cellular membranes. Saturation BRET studies can be highly informative because under conditions in which every energy donor is complexed with a suitable acceptor, the BRET signal should be maximal (Mercier et al., 2002). However, simple measurements of the extent of BRET signal are not inherently informative. For example, in heterodimer studies, if the lengths of the C-terminal tails of the two GPCRs are substantially different, then the distance between the BRET reporters attached to the C terminal tail is likely to be greater than for two GPCRs with very similar tail lengths. The effect of distance on resonance energy transfer signal (Eidne et al., 2002) might then result in a significantly greater signal for the latter pairing than the former, but it would be incorrect to use this to conclude that the latter pair formed a “better” or more high-affinity dimer. This limitation can be extended to homodimer analysis, because little is known about the orientation of the monomers in GPCR dimers. Thus, although the maximal signal produced when BRET-competent pairs of the α1A-1-adrenoceptor and the DOP receptor were coexpressed was less than for the two homodimer pairings, this does not inherently provide information on relative dimerization propensity. In contrast, the energy acceptor-to-donor ratio at which 50% of maximal BRET signal is achieved can provide such information because now 50% of the donor is in a positive BRET complex with acceptor. The α1A-1-adrenoceptor-DOP receptor pairing required a ratio some 40- to 75-fold higher that for either homodimer to achieve BRET 50. This is likely to be a generally applicable means of assessing GPCR heterodimerization selectivity. A series of reports have produced data consistent with interactions between coexpressed opioid and adrenoceptors, with particular focus on the β2- (Jordan et al., 2001) and α2A-adrenoceptors (Jordan et al., 2003). However, to our knowledge, this is the first study to provide quantitative data and suggests that such heterodimers will be uncommon species. In any circumstance in which two coexpressed GPCRs can be shown to form a heterodimer, it is inherently obvious that the two corresponding homodimers will also be present. The propensity of the heterodimers to form will be defined by the expression levels and mutual affinities, and studies such as these will help to illuminate the likelihood of significant levels of heterodimers in physiological settings.

We recently introduced the use of pairs of nonfunctional but potentially complementary GPCR-G protein fusion proteins (Carrillo et al., 2003). Herein, the first GPCR-G protein fusion contains a mutation in the G protein such that it cannot bind GTP and thus cannot be activated, whereas the second has mutations in the GPCR that prevent G protein activation but not the binding of ligands. Coexpression of a pair of such mutated fusions generated from a wild-type α1A-1-adrenoceptor–Gαq11 fusion protein resulted in reconstitution of agonist-stimulated [35S]GTPγS binding. These studies are unable to prove a direct interaction between the two copies of the GPCR but only that they are sufficiently close to allow functional interactions between the GPCR and G protein elements of the two constructs. This is not inherently different from the BRET and Tr-FRET studies, in which the constraints of the basis of energy transfer define proximity between the partner proteins but do not provide definitive proof of a direct interaction. However, the application of three distinct techniques in these studies, allied to previous data using both coimmunoprecipitation (Uberti et al., 2003) and FRET (Stanasila et al., 2003), combines to produce a convincing argument.

Although historically it was difficult to measure loading of [35S]GTPγS onto G proteins of the Gq/G11 family because of the high background signal provided by coexpressed Gq-family G proteins, the addition of end-of-assay immunocapture steps allows robust assays (Milligan, 2003). A number of GPCR-G protein fusions have been shown to interact with and activate endogenous G proteins as well as the G protein element of the fusion (Burt et al., 1998; Molinari et al., 2003). This was not a significant issue in the current studies. Little agonist-induced [35S]GTPγS binding was observed after expression of the α1A-1-adrenoceptor–Gly208AlaGαq11 fusion, despite the immunoaffinity step using an anti-Gαq antibody that immunoprecipitates endogenously expressed Gαq as well as the fusion protein. This indicates that...
the GPCR-G protein fusion proteins had little capacity to access and activate endogenously expressed G proteins.

A significant fraction of α1-adrenoceptors is present at intracellular locations in both transfected cell systems and in native tissues. This is particularly the case for the α1A-adrenoceptor (Hirasawa et al., 1997). BRET studies do not provide spatial information. Because both the luciferase and the fluorescent protein are attached to the C-terminal tail of the GPCRs, all that can be determined is that the signal is produced from locations inside the cell. Microscopy of HEK293 cells transfected to express α1A-1-adrenoceptor–GFP confirmed the intracellular location of a significant amount of the construct, and the addition of α1-adrenoceptor antagonists that fluoresce strongly when bound to receptor confirmed this location. The presence of the α1A-1-adrenoceptor intracellularly was not caused simply by the attachment of GFP to its C-terminal tail, because the distribution pattern of the untagged α1A-1-adrenoceptor also indicated a mixture of cell-surface and intracellular location. We have used previously Tr-FRET as a means to detect cell surface DOP receptors and to examine whether the dimerization status of the fraction of the receptors delivered to the cell surface was modified by agonist or inverse-agonist ligands (McVey et al., 2001). By applying the same approach to the α1A-1-adrenoceptor, the cell-surface fraction was shown to contain dimers/oligomers and that this was unaffected by the presence of agonist. Although the use of antiepitope tag antibodies linked to the Tr-FRET energy donors and acceptors restricted analysis in intact cells to cell surface dimers/oligomers, these can also be applied to isolated cell-membrane fractions. Homogenates of HEK293 cells transfected to express a combination of N-terminally FLAG- and c-myc–tagged forms of the α1A-1-adrenoceptor produced a strong Tr-FRET signal upon the addition of both Eu3+-labeled anti–c-myc and XL665-labeled anti-FLAG antibodies. Such homogenates were centrifuged through sucrose-density gradients to enrich membrane fractions based on buoyant density. When these fractions were used to monitor both [3H]prazosin binding sites and Tr-FRET, signals consistent with receptor dimers were distributed in two distinct peaks in the gradient, suggesting that the α1A-1-adrenoceptor exists as a dimeric/oligomeric complex in all membrane fractions in which it is present. The higher density peak seems to represent those α1A-1-adrenoceptors at the cell surface, because well-characterized markers of the plasma membrane were heavily enriched in this region, and this was also the location of the single observed peak of the β2-adrenoceptor that is endogenously expressed by HEK293 cells.

### TABLE 2

Ligand binding characteristics of C-terminal α1A-adrenoceptor splice variants

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[3H]prazosin Ki (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1A-1-AR</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>α1A-2A-AR</td>
<td>0.41 ± 0.12</td>
</tr>
<tr>
<td>α1A-3A-AR</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>α1A-7-AR + α1A-2A-AR</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>α1A-1-AR + α1A-3A-AR</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>α1A-2A-AR + α1A-3A-AR</td>
<td>0.35 ± 0.04</td>
</tr>
</tbody>
</table>

An important issue was to address whether heterodimers of α1A-adrenoceptor C-terminal splice variants could be produced and, if so, demonstrate whether the low-affinity binding site for prazosin that has been designated the α1L-adrenoceptor reflected such interactions. Suitably N-terminally tagged versions of each of α1A-2A- and α1A-3A-adrenoceptors produced Tr-FRET signals consistent with the presence of cell surface receptor homodimers. Similar results were obtained for combinations of both the α1A-2A- and α1A-3A-adrenoceptors with the α1A-1-adrenoceptor. Recent studies have provided support for transmembrane helix I as an important dimerization interface for the α1H-adrenoceptor (Carrillo et al., 2003; Stanasila et al., 2003) and that the C-terminal tail does not contribute (Stanasila et al., 2003). Because this also seems to be true for the α1A-1-adrenoceptor (Uberti et al., 2003), then they are consistent with the similar levels of Tr-FRET signals obtained in the splice-variant homodimer and heterodimer studies. Despite these clear interactions and evidence that these variants are coexpressed in tissues in which α1L-adrenoceptor pharmacology can be observed (Coge et al., 1999), ligand binding studies failed to uncover such pharmacology with coexpression of pairs of splice variants whether they were performed in intact cells or membrane preparations. Interactions between α1-adrenoceptor subtypes have also been indicated to be without effect on basic pharmacology (Uberti et al., 2003), but this must be tempered with appreciation that equal coexpression of both GPCRs with equivalent interaction affinity is only expected to generate the heterodimer to a level of 50% of the total number of ligand binding sites. Other splice variants of the human α1A-adrenoceptor are known, including truncated forms that do not produce the seventh transmembrane helix and C-terminal tail (Coge et al., 1999). Some of these have been reported to interact with the α1A-adrenoceptor and alter cell-surface delivery (Coge et al., 1999). Such data again rule out a key role for the C-terminal tail in α1A-adrenoceptor dimerization, but such interactions may contribute to α1L-adrenoceptor pharmacology. Many GPCRs have been shown to interact with a range of intracellular polypeptides (Milligan and White, 2001; Bockaert et al., 2003) and so regulate endocytosis, cell trafficking, and cell signaling. Such interactions may define α1-adrenoceptor pharmacology and would be restricted to cells and tissues that express the relevant interacting protein. Proteomic analysis provides a potential framework for such understanding (Bockaert et al., 2003). However, although C-terminal splice variants of the α1A-adrenoceptor can clearly form both homo- and heteromeric complexes, this, in itself, does not seem to generate a binding site with low affinity for prazosin.

### References


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