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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 $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-adrenoceptor homodimer interactions to be high affinity and some 75 times greater than interactions between the $\alpha_{1A\text{-}1}$-adrenoceptor and the δ opioid peptide receptor. Only a fraction of the $\alpha_{1A\text{-}1}$-adrenoceptors was at the plasma membrane of HEK293 cells at steady state. However, dimers of $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-adrenoceptor with differences limited to the C-terminal tail. Each of the $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2a}$, and $\alpha_{1A\text{-}3a}$-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

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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]GTPγS, 5′-*O*-(3-[^{35}S]thio)triphosphate; QAPB, BODIPY-FL prazosin; RQAPB, red BODIPY-FL prazosin; XL665, allophycocyanin; 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; Rocheville 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 μ-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 α_1-adrenoceptor pharmacology. This includes high-affinity binding of the antagonist prazosin (Piascik and Perez, 2001). Message encoding the α_{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 hypertrophy (Pool and Kirby, 2001). However, the presence of an α_1-adrenoceptor–like binding site with low affinity for prazosin, named the α_{1L}-adrenoceptor, has been shown in human prostate (Ford et al., 1997). Although there have been suggestions that the α_{1L}-adrenoceptor represents a distinct functional state of the α_{1A}-adrenoceptor (Ford et al., 1997), this remains unclear, and none of the classic human α_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 α_1-adrenoceptor grouping that is likely to correspond to this binding site (Fredriksson et al., 2003). It is thus possible that the α_{1L}-adrenoceptor corresponds to a heterodimer containing the α_{1A}-adrenoceptor. A number of both homo- and heterodimeric interactions between α_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 α_{1A}-adrenoceptor (Coge et al., 1999). A number of the human α_{1A}-adrenoceptor splice variants differ from the prototypic $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-adrenoceptor to provide means to analyze selectivity of GPCR heterodimerization, to assess interactions between C-terminal splice variants of the human α_{1A}-adrenoceptor, and to explore whether coexpressed combinations of these produced α_{1L}-adrenoceptor pharmacology.

Materials and Methods

Materials. Production and characterization of the anti-G_q/G_{11} antiserum CQ was described previously (Mitchell et al., 1993). Oligonucleotides were purchased from Thermohybaid (Ulm, Germany). All materials for tissue culture were supplied by Invitrogen (Paisley, Strathclyde, UK). [^{3}H]Prazosin (80 Ci/mmol) and [^{35}S]GTPγS (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 resonance 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 $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc involved PCR of the human $\alpha_{1A\text{-}1}$-adrenoceptor sequence. Using the amino-terminal primer 5′-AAA AGG TAC CAT GGT GTT TCT CTC GGG AAA TGC TTC-3′, a KpnI restriction sequence was introduced upstream of the coding sequence. Using the carboxyl-terminal primer 5′-AAA AGC GGC CGC GAC TTC CTC CCC GTT CTC ACT GAG GG-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′-AAG CGG CCG CTA CTT CGA AAG TTT ATG-3′ to introduce a NotI restriction sequence upstream of the coding sequence. The carboxyl-terminal primer 5′-GCG 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 $\alpha_{1A\text{-}1}$-adrenoceptor-GFP^2 involved PCR of the human $\alpha_{1A\text{-}1}$-adrenoceptor sequence using amino-terminal primer 5′-AAA AGG TAC CAT GGT GTT TCT CTC GGG AAA TGC TTC-3′ to introduce a KpnI restriction sequence upstream of the coding sequence. The carboxyl-terminal primer 5′-AAA AGG ATC CGA CTT CCT CCC CGT TCT CAC TGA GGG-3′ resulted in removal of the receptor stop codon and introduction of a BamH1 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 (DYKDDDDK) epitope tags were introduced immediately upstream of each of the human $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2a}$, and $\alpha_{1A\text{-}3a}$-adrenoceptor splice variants. The amino-terminal primers 5′-AAA AGG TAC CAT GGA CTA CAA GGA CGA CGA TGA TAA GGT GTT TCT CTC GGG AAA TGC TTC C-3′ or 5′-AAA AGG TAC CAT GGA ACA AAA ACT TAT TTC TGA AGA AGA TCT GGT GTT TCT CTC GGG AAA TGC 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 carboxyl-terminal primers were used: $\alpha_{1A\text{-}1}$-adrenoceptor, 5′-AAA AGG ATC CCT AGA CTT CCT CCC CGT TCT CAC TGA GGG-3′ incorporating a BamH1 site downstream of the coding sequence; $\alpha_{1A\text{-}2a}$-adrenoceptor, 5′-GGA CTC TAG ATC ATG AGG TCA AGA GAT CG-3′ incorporating an XbaI site downstream of the coding sequence; and $\alpha_{1A\text{-}3a}$-adrenoceptor, 5′-GGT CTC TAG ATC ATG TCA TGG GTG TGT G-3′ incorporating an XbaI site downstream of the coding sequence. All PCR fragments were subsequently cloned into pcDNA3.

Construction of the wild-type $\alpha_{1A\text{-}1}$-adrenoceptor–Gα_{11} fusion protein required PCR amplification of both $\alpha_{1A\text{-}1}$-adrenoceptor and Gα_{11}. PCR of the $\alpha_{1A\text{-}1}$-adrenoceptor used an amino-terminal primer 5′-TTA GGC AAG CTT GCC ACC ATG GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG GTG TTT CTC 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 GGC CGC TGA GGT CAA GAG ATC GAG ATC-3′, the receptor stop codon was removed, and a NotI restriction enzyme site introduced downstream of the receptor coding sequence. Gα_{11} was PCR-amplified using the amino-terminal primer 5′-A AGC ATT TCA GCG GCC GCA ACT CTG GAG TCC ATG ATG G-3′. This introduced a NotI restriction sequence upstream of the coding sequence. The carboxyl-terminal primer 5′-ACA GTT CTC GAG TCA CAC CAG GTT GTA 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 Leu^{132}Asp $\alpha_{1A\text{-}1}$-adrenoceptor-Gα_{11} was achieved by PCR amplification of two receptor minifragments: 1) using amino-terminal primer 5′- TAA GGA ATT CGC CAC CAT GGA CTA CAA GGA CGA CGA TGA CAA GGT GTT 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 Leu^{132}Asp substitution; and 2) using the amino-terminal primer 5′-GGT TGG GTA GCG GTC CGG GTA GCT CAC-3′, containing the Leu^{132}Asp substitution and the carboxyl-terminal primer 5′-A AGC ATT TCA GCG GCC GCA ACT CTG GAG TCC ATG ATG G-3′, which removed the stop codon and introduced a NotI 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′-TAA GGA ATT CGC CAC CAT GGA CTA CAA GGA CGA CGA TGA CAA GGT GTT TCT CTC GGG AAA TCG-3′ and 5′-A AGC ATT TCA GCG GCC GCA ACT CTG GAG TCC ATG ATG G-3′ to generate a single cDNA sequence containing the Leu^{132}Asp mutation in intracellular loop 2. This was then digested and substituted for the wild-type $\alpha_{1A\text{-}1}$-adrenoceptor in the $\alpha_{1A\text{-}1}$-adrenoceptor-Gα_{11} construct described above.

Construction of $\alpha_{1A\text{-}1}$-adrenoceptor-Gly^{208}Ala Gα_{11} was achieved through PCR amplification of Gly^{208}Ala Gα_{11} using a previously described (Carrillo et al., 2003) hamster α_{1b}-adrenoceptor-Gly^{208}Ala Gα_{11} fusion protein construct as a template. The amino-terminal primer 5′-A AGC ATT TCA GCG GCC GCA ACT CTG GAG TCC ATG ATG G-3′ was used to introduce a NotI site upstream of the coding sequence. The amino-terminal primer 5′-ACA GTT CTC GAG TCA CAC CAG GTT GTA CTC C-3′ introduced an XbaI restriction sequence downstream from the receptor coding sequence. This was then digested and substituted for wild-type Gα_{11} in the $\alpha_{1A\text{-}1}$-adrenoceptor-Gα_{11} construct described above.

Cell Culture. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g/l L-glutamine and 10% (v/v) newborn calf serum and incubated at 37°C with 5% CO_2. Chinese hamster ovary-K1 cells were maintained in Dulbecco's modified Eagle's medium-Ham's F-12 medium supplemented with 0.292 g/l L-glutamine and 10% (v/v) newborn calf serum and incubated at 37°C with 5% CO_2. Cells were grown to approximately 60% confluence before transfection using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions.

BRET^2 Assay. Cells were washed three times in phosphate-buffered saline (PBS) and then harvested in PBS supplemented with magnesium (0.1 g/l) and glucose (1 g/l). They were then counted on a hemocytometer, and approximately 700,000 cells were dispensed per well into a 96-well, white-walled culture plate (PerkinElmer). Compounds to be tested were dissolved in the same buffer as was used for cell resuspension and then were dispensed into the wells of the plate to achieve a final concentration of 10 μM. The plate was left standing for 5 min at 37°C to allow time for the ligands to bind to the expressed GPCR constructs. DeepBlueC (PerkinElmer) reagent was prepared in accordance with the manufacturer's directions and added to a final concentration of 10 μM. BRET^2 signals were then measured immediately in a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany), using 410 nm (band pass, 80 nm) to measure light emitted from DeepBlueC and a 515-nm (band pass, 30 nm) filter to measure light emitted from GFP^2. The extent of energy transfer was defined as the ratio of light intensity (515 nm) to light intensity (410 nm), with the ratio obtained from cells expressing the appropriate *R*-Luc construct alone defined as zero energy transfer.

Time-Resolved Fluorescence Resonance Energy Transfer. Time-resolved fluorescence resonance energy transfer was performed on intact HEK293 cells using Eu^{3+}-labeled anti–c-myc antibodies and XL665-labeled anti-FLAG antibodies as described previously (McVey et al., 2001).

Quantification of *R. reniformis* Luciferase- and GFP^2-Tagged GPCR Constructs. To quantify levels of expressed *R*-Luc– and GFP^2-tagged GPCRs in cells, a series of calibration curves were established for each individual GPCR construct. First, saturation radioligand binding was performed on membranes expressing either DOP–*R*-Luc, DOP-GFP^2, $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc, or $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2. The same membranes were then subject to serial dilution, and the fluorescence (monitored in a Victor^2 multilabel counter; PerkinElmer) or luminescence (Mithras LB 940; PerkinElmer) output (after addition of 5 μM *h*-coelenterazine) from each dilution point was determined. Fluorescence measurements were carried out in 386-well black-walled assay plates (Costar, Cambridge, MA), and luminescence readings were carried out in 96-well white-walled assay plates. For the luminescence assay, in which the signal output is time-dependent, plate readings were always performed exactly 30 min after the addition of *h*-coelenterazine. The protein content of each dilution point was then quantified using the BCA protein assay. In each case, background fluorescence/luminescence was subtracted using equivalent dilutions of nontransfected HEK293 cells. GPCR construct expression levels were then plotted against luminescence/fluorescence to determine the number of copies of *R*-Luc– and GFP^2-tagged GPCR constructs in transfected cell samples.

Radioligand Binding. Cells were washed three times with ice-cold PBS (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4). Cells were then detached from plates with PBS/0.5 mM EDTA, pelleted, and resuspended in ice-cold TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5) and lysed with two 10-s bursts in a Polytron homogenizer (Kinematica, Basel, Switzerland). The homogenate was centrifuged at 500*g* to remove unbroken cells and nuclei. The supernatant fraction was then centrifuged at 48,000*g* for 30 min, and the pellet was resuspended in TE buffer and stored at −80°C until use. [^{3}H]Prazosin binding studies were performed on such membrane preparations. Membrane protein (10 μg) was added to tubes containing 50 mM Tris-HCl, 1 mM EDTA, and 10 mM $MgCl_2$, pH 7.4, and [^{3}H]prazosin (variable concentrations) in the absence or presence of 10 μM unlabeled prazosin to define nonspecific binding at 30°C for 50 min. [^{3}H]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 $MgCl_2$, pH 7.4, and [^{3}H]diprenorphine (variable concentrations) in the absence or presence of 100 μM unlabeled naloxone as a competitor at 30°C for 50 min. In other studies, [^{3}H]prazosin binding assays were performed on intact cells in which approximately 400,000 cells were used for each assay point. In this case, incubation was at 37°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.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding was performed on membrane preparations containing 25 fmol of the various $\alpha_{1A\text{-}1}$-adrenoceptor–Gα_{11} fusion constructs as determined by saturation [^{3}H]prazosin binding studies. They were initiated by the addition of

membranes to an assay buffer (20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 50 nCi of [^{35}S]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 $MgCl_2$, 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 precleared with Pansorbin (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 [^{35}S]GTPγS was measured by liquid scintillation spectrometry (Liu et al., 2002).

Sucrose Density Gradient Preparation. Dishes (10 × 10 cm) were cotransfected with c-myc $\alpha_{1A\text{-}1}$-adrenoceptor and FLAG$\alpha_{1A\text{-}1}$-adrenoceptor, or, as a negative control, 5 × 10-cm dishes were singly transfected with c-myc $\alpha_{1A\text{-}1}$-adrenoceptor or FLAG$\alpha_{1A\text{-}1}$-adrenoceptor. Forty-eight hours later, these were harvested in PBS. At this point, the cell populations singly expressing c-myc $\alpha_{1A\text{-}1}$-adrenoceptor and FLAG$\alpha_{1A\text{-}1}$-adrenoceptor were mixed. Cells were pelleted by centrifugation at 1500 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 $MgCl_2$, and 1 mM EDTA plus a protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN). The homogenate was transferred to a centrifuge tube and overlayed with 2 ml of 80% sucrose solution. This was then further overlayed 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 H_2O 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-Eu^{3+} and anti-FLAG XL665 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 recentrifuged 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 E_{665}/E_{615} obtained from the mixed cell samples was subtracted from the ratio E_{665}/E_{615} obtained from coexpressed constructs to obtain a FRET reading minus background. Analysis of the distribution in such gradients of protein, the β_2-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 (Bourova 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 Fluor Apochromat objective lens (numerical aperture = 1.4) (Carl Zeiss Inc., Thornwood, NY). The following laser lines were used for excitation: 488 nm for GFP^2, and 543 nm for a red variant (RQAPB) of the fluorescent α_1-adrenoceptor antagonist ligand QAPB. The following Zeiss filter sets were used to detect the fluorescence of each fluorophore: BP505-530 for GFP^2, 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 GFP^2 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 GFP^2-tagged version of the human $\alpha_{1A\text{-}1}$-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 GFP^2 and RQAPB fluorescence emission intensity associated with each transfected cell.

Dual Hoechst and QAPB Epifluorescence Imaging. Cells transiently transfected to express the human $\alpha_{1A\text{-}1}$-adrenoceptor were rinsed several times in PBS and then incubated at 37°C with PBS supplemented with 10 nM concentrations of the green fluorescent α_1-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. QAPB/Hoechst-stained cells were imaged using a Diaphot 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 SnapHQ; 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 homo- and heterodimeric/oligomeric interactions between GPCRs 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 GFP^2 is the energy acceptor, however, has recently resulted in significant use of BRET^2 (Mercier et al., 2002; Ramsay et al., 2002). Forms of the human $\alpha_{1A\text{-}1}$-adrenoceptor tagged at the C terminus with either *R*-Luc or GFP^2 were generated and expressed transiently in HEK293 cells. The binding affinity of the α_1-adrenoceptor antagonist/inverse agonist [^{3}H]prazosin to these constructs in cell membrane preparations was not significantly different (K_d for $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc = 0.71 ± 0.18 nM; K_d for $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 = 0.95 ± 0.10 nM) but was some 3-fold lower than the unmodified human $\alpha_{1A\text{-}1}$-adrenoceptor (K_d = 0.28 ± 0.04 nM).

Coexpression of $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc and $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 in HEK293 cells followed by the addition of DeepBlueC resulted in a BRET^2 signal consistent with these two forms of the receptor forming a constitutive complex (Fig. 1). Addition of adrenaline (10 μM) did not modify the BRET^2 signal (Fig. 1), indicating that agonist binding did not alter

this interaction. Equally, the addition of prazosin (10 μM) did not modify the BRET^2 signal (data not shown).

A key requirement in GPCR dimerization studies is to provide evidence of selectivity/specificity of the observed interactions. It is frequently difficult to produce convincingly negative data, and this may indicate that GPCRs have a natural propensity to interact (Salim et al., 2002). We have previously used BRET^2 to demonstrate that the DOP receptor can form a constitutive dimeric/oligomeric complex (Ramsay et al., 2002). This was confirmed after coexpression of DOP–*R*-Luc and DOP-GFP^2 (Fig. 1). The BRET^2 signal generated after coexpression of the pair of DOP receptor constructs was higher than that produced by coexpression of the $\alpha_{1A\text{-}1}$-adrenoceptor BRET^2 pair (Fig. 1), despite antagonist [^{3}H]ligand binding studies indicating that the $\alpha_{1A\text{-}1}$-adrenoceptor pair could be expressed at substantially higher levels than the DOP receptor pairing (see below). We thus tested whether an interaction could be observed between the $\alpha_{1A\text{-}1}$-adrenoceptor and the DOP receptor. Coexpression of either $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc with DOP-GFP^2 or DOP–*R*-Luc with $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 did produce BRET^2 energy transfer signals upon the addition of DeepBlueC. These BRET^2 signals are consistent with a heterodimeric interaction between this receptor pair. However, the signals were substantially smaller than for either receptor homodimer pairing (Fig. 1). These were unaffected by the addition of a combination of adrenaline and the synthetic enkephalin D-Ala^2,D-Leu^5-enkephalin, which is an agonist at the DOP receptor (both at 10 μM), and were not substantially different whether the DOP receptor or the $\alpha_{1A\text{-}1}$-adrenoceptor acted as energy donor (Fig. 1).

Although BRET^2 signals were obtained upon coexpression of the DOP receptor and $\alpha_{1A\text{-}1}$-adrenoceptor constructs we wished to explore if these represented high affinity interactions. Resonance energy transfer signals are exquisitely sensitive to small differences in the distance between, and the orientation of, the energy donor and acceptor species (Eidne et al., 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 $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc, DOP–*R*-Luc, $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2, or DOP-GFP^2 and correlated these with expression levels monitored by saturation binding studies using the antagonists [^{3}H]prazosin ($\alpha_{1A\text{-}1}$-adrenoceptor) or [^{3}H]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 $\alpha_{1A\text{-}1}$-adrenoceptor constructs. Although unexpected, similar observations have been noted previously in direct comparisons between equivalently tagged forms of the β_1- and β_2-adrenoceptors (Mercier et al., 2002). Saturation BRET^2 experiments (Mercier et al., 2002) were then performed. In these, the ratio of the energy acceptor GPCR-GFP^2 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 $\alpha_{1A\text{-}1}$-adrenoceptor pair, BRET^2 signals increased as a hyperbolic function with increasing acceptor-to-donor ratio, reaching an asymptote of 0.38 $\pm$ 0.011 (n = 3) (Fig. 3A). Half-maximal BRET signal (BRET$_{50}$) was achieved at an estimated $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2/$\alpha_{1A\text{-}1}$-adrenoceptor–*R*-

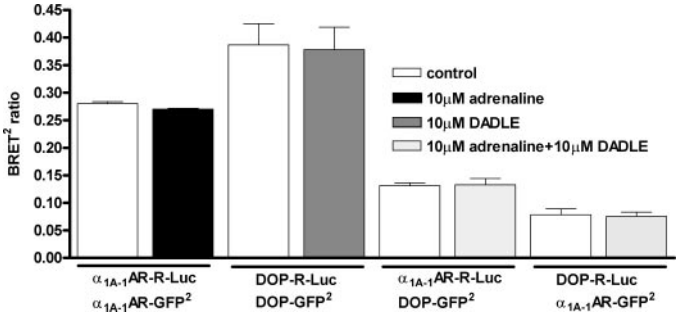

Fig. 1. Homo- and heterointeractions of $\alpha_{1A\text{-}1}$-adrenoceptor and DOP-opioid receptors. BRET^2-competent combinations of the human $\alpha_{1A\text{-}1}$-adrenoceptor and the DOP opioid receptor were expressed transiently in HEK293 cells. Constitutive interactions (□) and the effects on this of agonist treatment (all other bar fills) were measured after the addition of DeepBlueC.

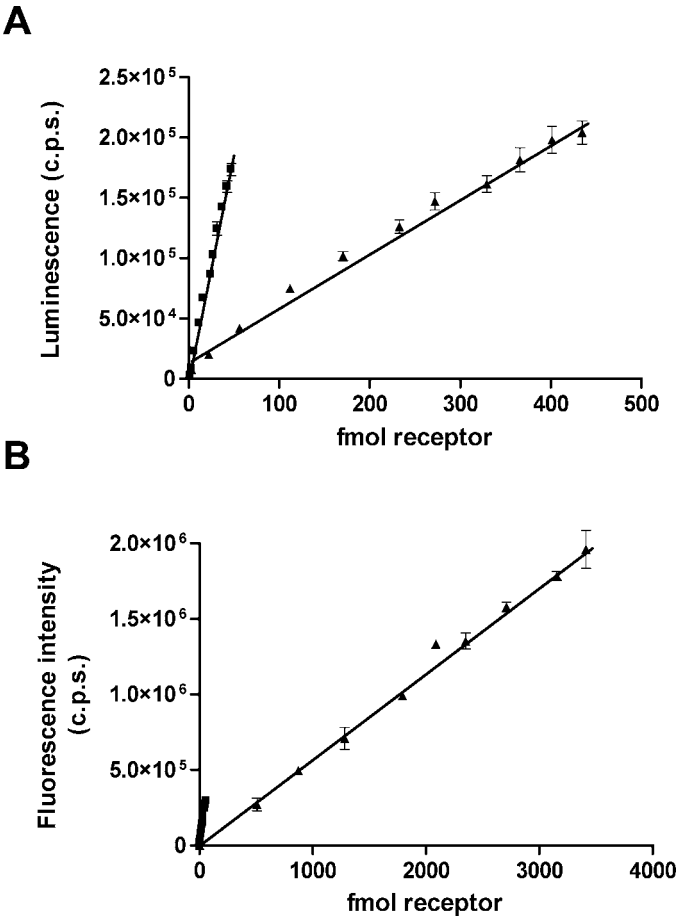

Fig. 2. Correlation of fluorescence and luminescence signals with expression levels of BRET^2-competent constructs. $\alpha_{1A\text{-}1}$-Adrenoceptor–*R*-Luc (A, ▲), DOP–*R*-Luc (A, ■), $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 (B, ▲), or DOP-GFP^2 (B, ■) were expressed in HEK293 cells. Expression levels in varying amounts of membranes were monitored by the specific binding of concentrations of [^{3}H]prazosin ($\alpha_{1A\text{-}1}$-adrenoceptor) or [^{3}H]diprenorphine (DOP receptor) shown to be close to saturating. Luminescence signals after addition of coelenterazine (A) or fluorescence signals (B) were monitored as described under *Materials and Methods*.

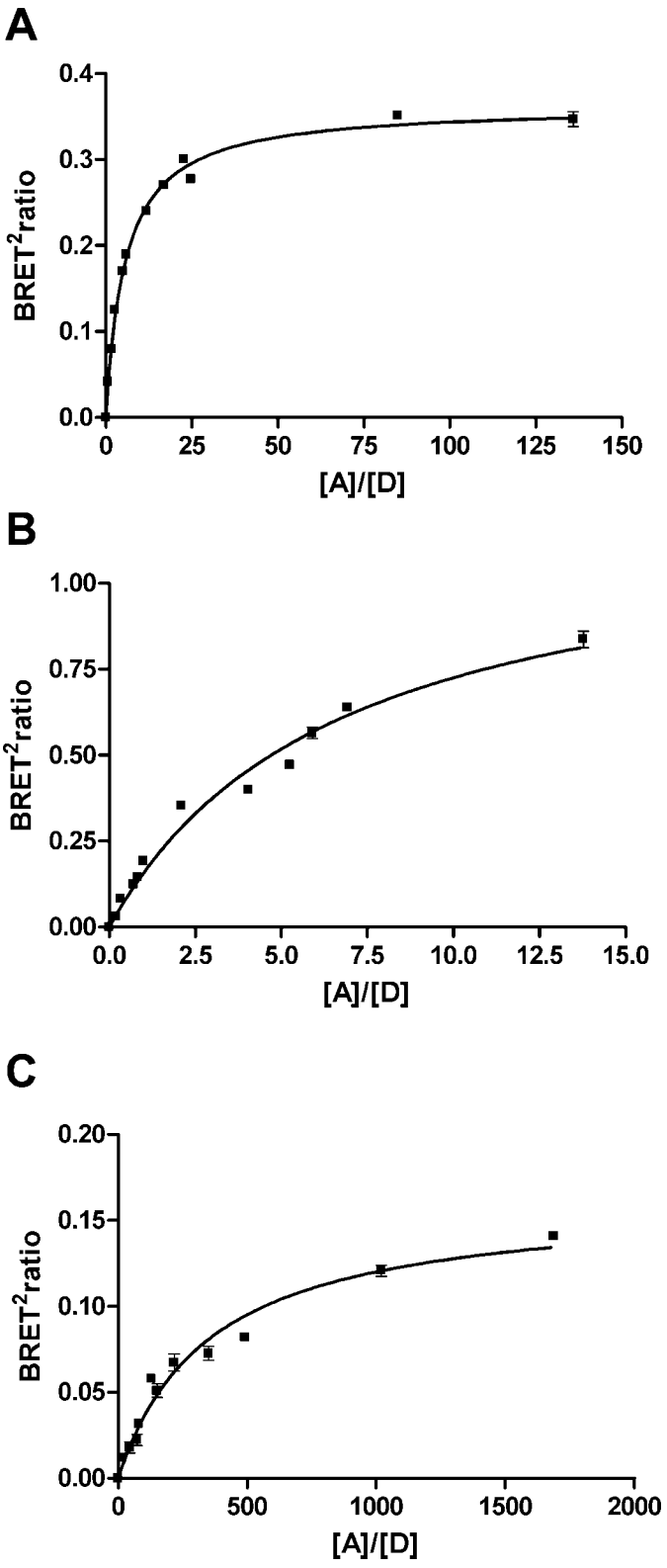

Fig. 3. Saturation BRET^2 studies show that heteromeric $\alpha_{1A\text{-}1}$-adrenoceptor–DOP receptor interactions are of low affinity. Varying amounts of $\alpha_{1A\text{-}1}$-adrenoceptor–*R*-Luc and $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 (A), DOP–*R*-Luc and DOP-GFP^2 (B), or DOP–*R*-Luc + $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 (C) were expressed in HEK293 cells, and their expression ratios were calculated as in Fig. 2. BRET^2 studies were then performed after addition of DeepBlueC. Energy transfer signals are plotted against the expression ratios of energy acceptor (GPCR-GFP^2) [A] and energy donor (GPCR–*R*-Luc) [D]. Absolute levels of the energy donor varied between 0.2 and 8 pmol/mg (A), 0.1 and 0.3 pmol/mg (B), and 0.2 and 1.0 pmol/mg (C).

Luc ratio of 4.56 ± 0.70, consistent with a high-affinity interaction. Equivalent studies using the DOP receptor BRET^2 pair also generated a hyperbolic function with asymptote of 1.14 ± 0.071 (n = 3) (Fig. 3B) and a measured $BRET_{50}$ acceptor-donor ratio of 9.8 ± 3.1. The ability to express substantially higher levels of the $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 as energy acceptor. In saturation BRET^2 studies, the energy transfer signal between DOP–*R*-Luc and $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 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 $BRET_{50}$ 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 $\alpha_{1A\text{-}1}$-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 Ca^{2+}-mobilizing G protein G_{11} was linked in-frame to the C-terminal tail of the $\alpha_{1A\text{-}1}$-adrenoceptor. This construct was expressed in HEK293 cells, and membranes were prepared. The binding affinity of [^{3}H]prazosin to this construct was similar (0.29 ± 0.02 nM, n = 3) to the wild-type $\alpha_{1A\text{-}1}$-adrenoceptor. Membrane amounts containing 25 fmol of [^{3}H]prazosin binding sites were used in [^{35}S]GTPγS binding assays performed in the absence or presence of the α_1-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 Gα_{11} and counted (Fig. 4). Little binding of [^{35}S]GTPγS was observed in the absence of agonist, indicating, as shown previously for the hamster α_{1B}-adrenoceptor (Carrillo et al., 2002), limited constitutive capacity of the human $\alpha_{1A\text{-}1}$-adrenoceptor to activate Gα_{11}. The presence of phenylephrine, however, resulted in a large stimulation of [^{35}S]GTPγS binding in the immunoprecipitate (Fig. 4). Gly^{208}Ala Gα_{11} is unable to bind GTP or its analogs (Carrillo et al., 2002, 2003). When Gly^{208}Ala Gα_{11} was linked in-frame to the $\alpha_{1A\text{-}1}$-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 [^{3}H]prazosin binding sites were used in [^{35}S]GTPγS binding studies as for the wild-type fusion protein, phenylephrine produced only a very small increase in [^{35}S]GTPγS binding (Fig. 4). Mutation of the hydrophobic Leu^{132} residue in the second intracellular loop of the $\alpha_{1A\text{-}1}$-adrenoceptor to aspartic acid also resulted in the loss of phenylephrine stimulation of [^{35}S]GTPγS binding when this form of the receptor was coupled to wild-type Gα_{11} (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 $\alpha_{1A\text{-}1}$-adrenoceptor–Gα_{11} fusion proteins were coexpressed in HEK293 cells and membranes containing 25 fmol of [^{3}H]prazosin binding sites used in [^{35}S]GTPγS binding assays, phenylephrine-induced binding of the nucleotide was reconstituted (Fig. 4). Agonist stimulation of [^{35}S]GTPγS 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 $\alpha_{1A\text{-}1}$-adrenoceptor dimers, and indeed, a number of studies have shown significant populations of intracellular α_1-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 $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 construct was not located at the plasma membrane. Although excluded from the nucleus, internal membranes displayed strong GFP^2 fluorescence (Fig. 5A). Addition of a red variant (RQAPB) of the fluorescent α_1-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 GFP^2-tagged $\alpha_{1A\text{-}1}$-adrenoceptor. The significant intracellular accumulation of the expressed $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 construct did not result simply from the addition of GFP^2. When HEK293 cells were transfected to express the isolated human $\alpha_{1A\text{-}1}$-adrenoceptor, the addition of QAPB, which fluoresces in the green region of the spectrum, identified substantial levels of intracellular receptor as well as some degree of cell-surface localization (Fig. 5C). Despite this, the addition of phenylephrine to HEK293 cells expressing the $\alpha_{1A\text{-}1}$-adrenoceptor and loaded with the Ca^{2+} indicator dye Fura-2 confirmed that the agonist was able to cause elevation of $[Ca^{2+}]_i$ (Fig. 5D). This was not observed in mock-transfected cells (data not shown).

To examine dimerization/oligomerization specifically of the population of the $\alpha_{1A\text{-}1}$-adrenoceptor that did reach the cell surface, we used Tr-FRET (McVey et al., 2001; Carrillo et al., 2003). Forms of the $\alpha_{1A\text{-}1}$-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 Eu^{3+}-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 Eu^{3+}(Fig. 6). Energy transfer was not produced when only the Eu^{3+}-labeled anti–c-myc antibodies were added or if HEK293 cells separately expressing the FLAG or c-myc tagged forms of the $\alpha_{1A\text{-}1}$-adrenoceptor were mixed before the addition of the antibodies (Fig. 6). As noted in the $BRET^2$ studies, the addition of adrenaline (10 μM) did not alter the energy transfer signal (Fig. 6). As with the $BRET^2$ studies, it was important to use a GPCR that has only limited interactions with the $\alpha_{1A\text{-}1}$-adrenoceptor. We have shown previously only weak interactions at the cell surface between the α_{1B}-adrenoceptor and the histamine H1 receptor (Carrillo et al., 2003). Coexpression of N-terminally tagged forms of the $\alpha_{1A\text{-}1}$-adrenoceptor and the histamine H1 receptor resulted in a very limited Tr-FRET signal upon the addition of the combination of Eu^{3+}-labeled anti–c-myc and XL665-labeled anti-FLAG antibodies (Fig. 6).

To determine whether the $\alpha_{1A\text{-}1}$-adrenoceptors located in in-

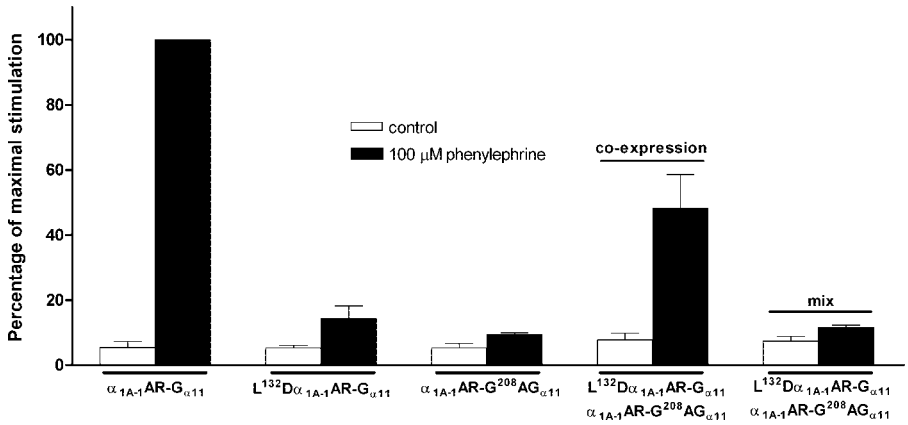

Fig. 4. Reconstitution of agonist-stimulated [^{35}S]GTPγS binding by coexpression of a pair of nonfunctional $\alpha_{1A\text{-}1}$-adrenoceptor–Gα_{11} fusion proteins. Fusion proteins containing the wild-type sequences of the $\alpha_{1A\text{-}1}$-adrenoceptor and Gα_{11}, Leu^{132}Asp$\alpha_{1A\text{-}1}$-adrenoceptor and wild-type Gα_{11}, or wild-type $\alpha_{1A\text{-}1}$-adrenoceptor and Gly^{208}Ala Gα_{11} were expressed in HEK293 cells. Leu^{132}Asp $\alpha_{1A\text{-}1}$-adrenoceptor–Gα_{11} and $\alpha_{1A\text{-}1}$-adrenoceptor–Gly^{208}AlaGα_{11} were also coexpressed or expressed in separate cell populations, which were then mixed. Cell membranes were produced, and saturation [^{3}H]prazosin binding studies were performed. Membrane amounts containing 25 fmol of [^{3}H]prazosin binding sites were used in [^{35}S]GTPγS binding studies in the absence (□) or presence (■) of phenylephrine (100 μM). At the termination of incubation samples were immunoprecipitated with the anti-Gα_{11}/Gα_q antiserum CQ (Mitchell et al., 1993) and counted. Data represent means ± S.E.M. from three independent experiments.

TABLE 1

The binding of agonist and antagonist ligands to $\alpha_{1A\text{-}1}$-adrenoceptor-Gα_{11} fusion proteins

Fusion proteins incorporating both wild-type and point mutant forms of the human $\alpha_{1A\text{-}1}$-adrenoceptor and Gα_{11} that result in a lack of information transfer from receptor to G protein in response to agonist ligands were expressed transiently in HEK293 cells and membranes prepared. The binding affinity of [^{3}H]prazosin and both phenylephrine and adrenaline was then estimated from ligand binding studies.

Construct	[^{3}H]Prazosin K_d	Phenylephrine K_i	Adrenaline K_i
	nM	*μM*	*μM*
WT$\alpha_{1A\text{-}1}$-Gα_{11}	0.29 ± 0.02	15.8 ± 9.9	4.0 ± 1.0
L^{132}D$\alpha_{1A\text{-}1}$-Gα_{11}	0.24 ± 0.03	30.8 ± 12.0	4.2 ± 0.9
$\alpha_{1A\text{-}1}$-G^{208}AGα_{11}	0.27 ± 0.04	28.3 ± 12.7	6.3 ± 2.1

WT, wild type.

tracellular membranes were also present as dimers/oligomers, HEK293 cells transfected to express a combination of FLAG- and c-myc–tagged forms of the $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-adrenoceptor. The denser gradient fractions containing the $\alpha_{1A\text{-}1}$-adrenoceptor were also enriched with both the oubain-sensitive Na^+/K^+ ATPase (Fig. 7) and adenylyl 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 β_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 α_{1A}-adrenoceptor have been reported to occur (Coge et al., 1999). $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2a}$, and $\alpha_{1A\text{-}3a}$ 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 α_{1L} pharmacology, an α_1-adrenoceptor binding site with significantly lower affinity for prazosin than other α_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 $\alpha_{1A\text{-}2a}$ and $\alpha_{1A\text{-}3a}$ forms alters the length and sequence of the C-terminal tail from that of the $\alpha_{1A\text{-}1}$ 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–$\alpha_{1A\text{-}1}$-adrenoceptor + c-myc–$\alpha_{1A\text{-}1}$-adrenoceptor, FLAG–$\alpha_{1A\text{-}2a}$-adrenoceptor + c-myc–$\alpha_{1A\text{-}2a}$-adrenoceptor, and FLAG–$\alpha_{1A\text{-}3a}$-adrenoceptor + c-myc–$\alpha_{1A\text{-}3a}$-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 μM) did not alter the energy transfer signal (Fig. 8). Equally, coexpression of the c-myc–$\alpha_{1A\text{-}1}$-adrenoceptor with either FLAG–$\alpha_{1A\text{-}2a}$-adrenoceptor or FLAG–$\alpha_{1A\text{-}3a}$-adrenoceptor resulted in production of a similar level of energy transfer signal, consistent with constitutive heterodimerization (Fig. 8). Again, the addition of adrenaline (10 μM) did not alter the energy transfer signals (Fig. 8). Equally, coexpression of FLAG–$\alpha_{1A\text{-}2a}$-adrenoceptor and c-myc–$\alpha_{1A\text{-}3a}$-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 $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2a}$, and $\alpha_{1A\text{-}3a}$ 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 α_{1L}-adrenoceptor binding site (data not shown).

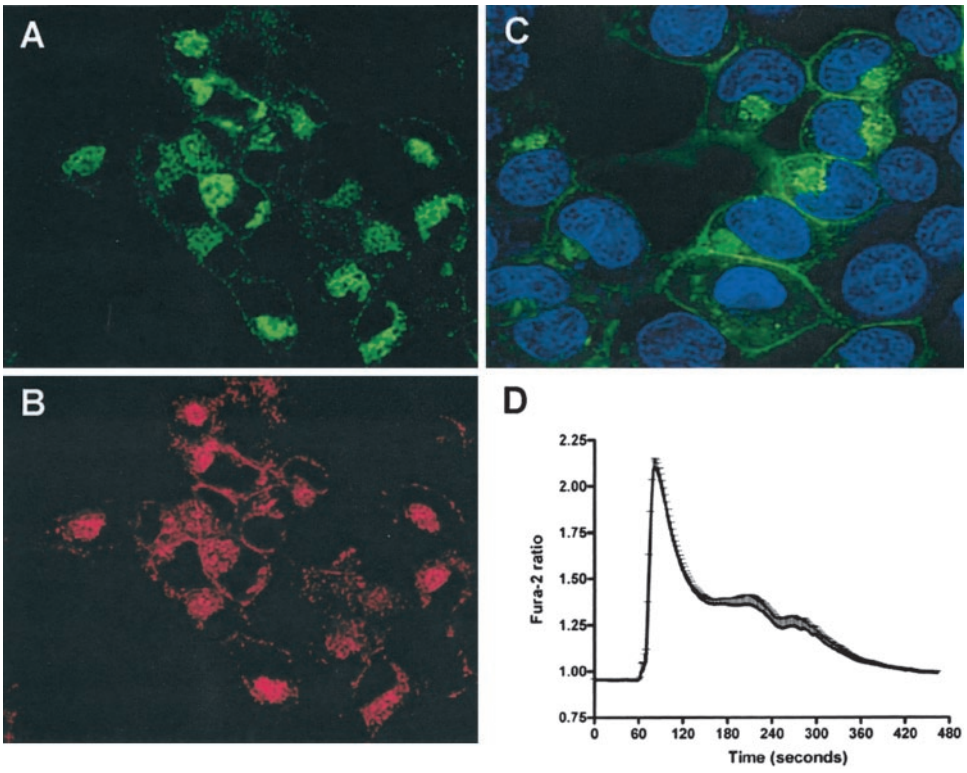

Fig. 5. Only a fraction of $\alpha_{1A\text{-}1}$-adrenoceptors are present at the plasma membrane of HEK293 cells. $\alpha_{1A\text{-}1}$-Adrenoceptor–GFP^2 was expressed transiently in HEK293 cells. The fluorescent α_1-adrenoceptor antagonist RQAPB was added, and the distribution of GFP^2 (A) and RQAPB (B) was monitored. The $\alpha_{1A\text{-}1}$-adrenoceptor was expressed transiently in HEK293 cells (C), and its distribution was monitored after the addition of QAPB (green). Hoechst 33342 (blue) was used in parallel to identify cell nuclei. D, phenylephrine (10 μM) was added to HEK293 cells expressing the $\alpha_{1A\text{-}1}$-adrenoceptor and loaded with Fura-2. The elevation of $[Ca^{2+}]_i$ was then recorded. Data represent mean ± S.E.M. of data analyzed from 15 separate cells.

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 β_2-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-

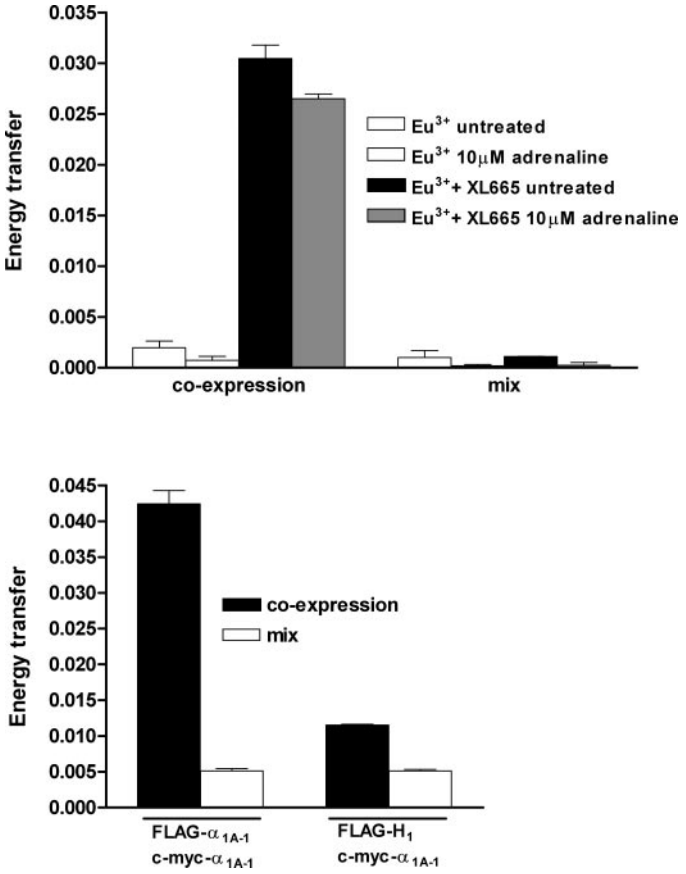

Fig. 6. Detection of cell surface $\alpha_{1A\text{-}1}$-adrenoceptor dimers using Tr-FRET. A, HEK293 cells were transfected to coexpress or separately express N-terminally FLAG- and c-myc–tagged forms of the $\alpha_{1A\text{-}1}$-adrenoceptor. Cells were harvested, and either Eu^{3+}-labeled anti–c-myc 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–$\alpha_{1A\text{-}1}$-adrenoceptor was coexpressed with FLAG-tagged forms of either the $\alpha_{1A\text{-}1}$-adrenoceptor or the histamine H1 receptor. In parallel, cells were transfected to express only c-myc–$\alpha_{1A\text{-}1}$-adrenoceptor, the FLAG–$\alpha_{1A\text{-}1}$-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 Eu^{3+}-labeled anti–c-myc antibodies and XL665-labeled anti-FLAG antibodies.

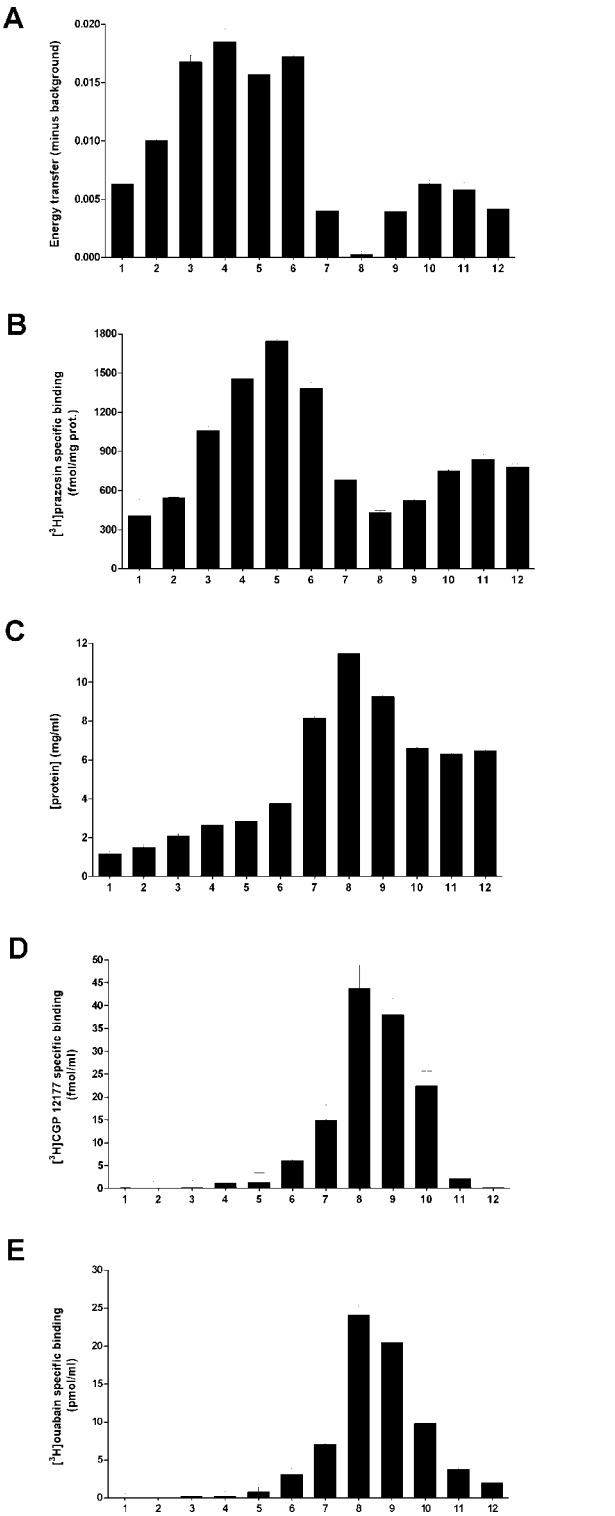

Fig. 7. The $\alpha_{1A\text{-}1}$-adrenoceptor exists as dimers/oligomers in multiple cellular locations. HEK293 cells were transfected to coexpress N-terminally FLAG- and c-myc–tagged forms of the $\alpha_{1A\text{-}1}$-adrenoceptor. Cells were harvested and homogenized. Aliquots of the homogenate 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, Eu^{3+}-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 [^{3}H]prazosin binding (B), protein (C), [^{3}H]CGP12177 binding (D), and the binding of [^{3}H]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 α_{1b}-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 terminial 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 α_{1A-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 α_{1A-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 β_2- (Jordan et al., 2001) and α_{2A}-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 α_{1A-1}-adrenoceptor–Gα_{11} fusion protein resulted in reconstitution of agonist-stimulated [^{35}S]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 [^{35}S]GTPγS onto G proteins of the G_q/G_{11} family because of the high background signal provided by coexpressed G_i-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 [^{35}S]GTPγS binding was observed after expression of the α_{1A-1}-adrenoceptor–Gly^{208}AlaGα_{11} fusion, despite the immunocapture step using an anti-Gα_{11}/Gα_q antiserum that immunoprecipitates endogenously expressed Gα_{11}/Gα_q as well as the fusion protein. This indicates that

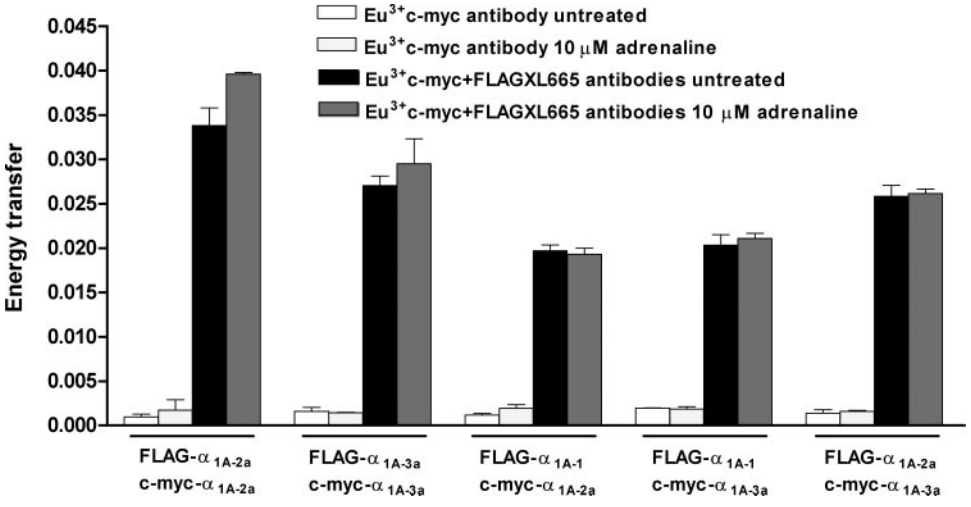

Fig. 8. C-terminal splice variants of the human α_{1A-1}-adrenoceptor form homo- and heterodimers at the surface of HEK293 cells. HEK293 cells were transfected to coexpress N-terminally FLAG- and c-myc–tagged forms of combinations of the α_{1A-1}, α_{1A-2a}, and α_{1A-3a}-adrenoceptors. Intact cells were used to measure Tr-FRET in cells coexpressing FLAG-α_{1A-2a} + c-myc α_{1A-2a}, FLAG-α_{1A-3a} + c-myc α_{1A-3a}, FLAG-α_{1A-1}+ c-myc α_{1A-2a}, FLAG-α_{1A-1} + c-myc α_{1A-3a}, or FLAG-α_{1A-2a} + c-myc α_{1A-3a}. Experiments were performed by the addition of only Eu^{3+}-labeled anti–c-myc antibodies or Eu^{3+}-labeled anti–c-myc antibodies and XL665-labeled anti-FLAG antibodies in the absence or presence of adrenaline (10 μM).

the GPCR-G protein fusion proteins had little capacity to access and activate endogenously expressed G proteins.

A significant fraction of α_1-adrenoceptors is present at intracellular locations in both transfected cell systems and in native tissues. This is particularly the case for the α_{1A}-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 $\alpha_{1A\text{-}1}$-adrenoceptor–GFP^2 confirmed the intracellular location of a significant amount of the construct, and the addition of α_1-adrenoceptor antagonists that fluoresce strongly when bound to receptor confirmed this location. The presence of the $\alpha_{1A\text{-}1}$-adrenoceptor intracellularly was not caused simply by the attachment of GFP^2 to its C-terminal tail, because the distribution pattern of the untagged $\alpha_{1A\text{-}1}$-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 $\alpha_{1A\text{-}1}$-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 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 $\alpha_{1A\text{-}1}$-adrenoceptor produced a strong Tr-FRET signal upon the addition of both Eu^{3+}-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 [^{3}H]prazosin binding sites and Tr-FRET, signals consistent with receptor dimers were distributed in two distinct peaks in the gradient, suggesting that the $\alpha_{1A\text{-}1}$-adrenoceptor exists as a dimeric/oligomeric complex in all membrane fractions in which it is present. The higher density peak seems to represent those $\alpha_{1A\text{-}1}$-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 β_2-adrenoceptor that is endogenously expressed by HEK293 cells.

TABLE 2

Ligand binding characteristics of C-terminal α_{1A}-adrenoceptor splice variants

C-terminal splice variants of the human α_{1A}-adrenoceptor were expressed either individually or in combination in HEK293 cells. Membranes were prepared, and saturation [^{3}H]prazosin binding studies were performed. Data represent means ± S.E.M. from three independent experiments.

Receptor	[^{3}H]prazosin K_d
	nM
$\alpha_{1A\text{-}1}$-AR	0.28 ± 0.04
$\alpha_{1A\text{-}2a}$-AR	0.41 ± 0.12
$\alpha_{1A\text{-}3a}$-AR	0.32 ± 0.07
$\alpha_{1A\text{-}1}$-AR + $\alpha_{1A\text{-}2a}$-AR	0.32 ± 0.03
$\alpha_{1A\text{-}1}$-AR + $\alpha_{1A\text{-}3a}$-AR	0.28 ± 0.05
$\alpha_{1A\text{-}2a}$-AR + $\alpha_{1A\text{-}3a}$-AR	0.35 ± 0.04

An important issue was to address whether heterodimers of α_{1A}-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 α_{1L}-adrenoceptor reflected such interactions. Suitably N-terminally tagged versions of each of $\alpha_{1A\text{-}2a}$- and $\alpha_{1A\text{-}3a}$-adrenoceptors produced Tr-FRET signals consistent with the presence of cell surface receptor homodimers. Similar results were obtained for combinations of both the $\alpha_{1A\text{-}2a}$- and $\alpha_{1A\text{-}3a}$-adrenoceptors with the $\alpha_{1A\text{-}1}$-adrenoceptor. Recent studies have provided support for transmembrane helix I as an important dimerization interface for the α_{1B}-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 $\alpha_{1A\text{-}1}$-adrenoceptor (Uberti et al., 2003), then they are consistent with the similar levels of Tr-FRET signals obtained in the splice-variant homo- and heterodimer studies. Despite these clear interactions and evidence that these variants are coexpressed in tissues in which α_{1L}-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 α_1-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 two 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 α_{1A}-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 $\alpha_{1A\text{-}1}$-adrenoceptor and alter cell-surface delivery (Coge et al., 1999). Such data again rule out a key role for the C-terminal tail in α_{1A}-adrenoceptor dimerization, but such interactions may contribute to α_{1L}-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 α_{1L}-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 α_{1A}-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.

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