Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences

Douglas RAMSAY*, Elaine KELLETT*, Mary McVEY*, Stephen REES† and Graeme MILLIGAN*[1]

*Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and †Cellular HTS and Technologies, Molecular Screening Department, GlaxoSmithKline, Gunnels Wood Road, Stevenage SG1 2NY, U.K.

Homo- and hetero-oligomerization of G-protein-coupled receptors (GPCRs) were examined in HEK-293 cells using two variants of bioluminescence resonance energy transfer (BRET). BRET^2 (a variant of BRET) offers greatly improved separation of the emission spectra of the donor and acceptor moieties compared with traditional BRET. Previously recorded homo-oligomerization of the human δ-opioid receptor was confirmed using BRET^2. Homo-oligomerization of the κ-opioid receptor was observed using both BRET techniques. Both homo- and hetero-oligomers, containing both δ- and κ-opioid receptors, were unaffected by the presence of receptor ligands. BRET detection of opioid receptor homo- and hetero-oligomers required expression of 50000–100000 copies of the receptor energy acceptor construct per cell. The effectiveness of δ–κ-opioid receptor hetero-oligomer formation was as great as for homomeric interactions. The capacity of the two opioid receptors to form oligomeric complexes with the β_2-adrenoceptor was also assessed. Although such interactions were detected, at least 250000 copies per cell of the energy acceptor were required. Requirement for high levels of receptor expression was equally pronounced in attempts to measure hetero-oligomer formation between the κ-opioid receptor and the thyrotropin-releasing hormone receptor-1. These studies indicate that constitutively formed homo- and hetero-oligomers of opioid receptor subtypes can be detected in living cells containing less than 100000 copies of the receptors. However, although hetero-oligomeric interactions between certain less closely related GPCRs can be detected, they appear to be of lower affinity than homo- or hetero-oligomers containing closely related sequences. Interactions recorded between certain GPCR family members in heterologous expression systems are likely to be artefacts of extreme levels of overexpression.

Key words: adrenaline, dimerization, energy transfer, G-protein, opioid.

INTRODUCTION

G-protein-coupled receptors (GPCRs) comprise one of the largest gene families in the human genome [1,2]. They are also the most successfully exploited set of targets for therapeutic drug intervention in disease [1]. Each GPCR is likely to have a similar overall topology, with seven transmembrane helices linked by a series of extra- and intra-cellular loops [3,4]. In the recent past, a considerable body of evidence has accumulated to indicate that GPCRs can exist as dimeric or oligomeric species. This area has been reviewed extensively [5–7]. Early work in this area relied on co-immunoprecipitation of differentially epitope-tagged GPCR species [8–12]. These studies have been complemented by analysis of ligand binding [13] and, to some degree, supplanted by biophysical studies that use resonance energy transfer approaches [14–20]. As well as interaction between multiple copies of the same gene product to produce homo-oligomers [8–10,14–17], there is growing evidence that GPCRs derived from different genes can interact to form hetero-oligomers [11,12,18,19,21,22]. Interest in this area was galvanized by recognition that production of a functional $GABA_B$ receptor ($GABA_BR$, where GABA is γ-aminobutyric acid) required the co-expression of two distinct, but significantly related, GPCRs, $GABA_BR1$ and $GABA_BR2$ [23]. Although a series of suggestions have been made in relation to the possible mechanisms of oligomerization and the interfaces of GPCRs that may contribute to this [8,24–26], clear unanimity in view is currently lacking. An attractive proposition is that more closely related GPCRs are more likely to interact with one another. However, although certain negative controls are incorporated into many studies of GPCR oligomerization, this has not been investigated in a systematic manner. Furthermore, it is often unclear when using heterologous expression systems what levels of expression of the GPCRs are required to observe their interaction.

We have recently used combinations of time-resolved fluorescence resonance energy transfer ('trFRET') and bioluminescence resonance energy transfer (BRET) to explore homo-oligomerization of the δ-opioid receptor [16]. We now utilize two variants of BRET to explore both homo- and hetero-oligomerization of opioid receptor subtypes and to ascertain if such interactions occur more readily than interactions of the opioid receptors with GPCRs with lower sequence similarity.

Abbreviations used: BRET, bioluminescence resonance energy transfer; DADLE, [D-Ala^2,D-Leu^5]enkephalin; eYFP, enhanced yellow fluorescent protein; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GABA, γ-aminobutyric acid; $GABA_BR$, $GABA_B$ receptor; GFP, green fluorescent protein; GNTI, 5′-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2′,3′-indolomorphinan; GPCR, G-protein-coupled receptor; Aib, α-aminoisobutyric acid; ICI174864, *N*, *N*-diallyl-Tyr-Aib-Aib-Phe-Leu; ICI199441, 2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-phenyl-2-(1-pyrrolidinyl)-ethyl]acetamide; SST, somatostatin; SSTR, SST receptor; TRHR-1, thyrotropin-releasing hormone receptor-1.

[1] To whom correspondence should be addressed (e-mail g.milligan@bio.gla.ac.uk).

EXPERIMENTAL

Materials

All materials for tissue culture were supplied by Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). [^{3}H]Diprenorphine (66 Ci/mmol), [^{3}H]naltrindole (33 Ci/mmol) and [^{3}H]dihydroalprenolol (40 Ci/mmol) were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Coelenterazine was from Prolume (Pittsburgh, PA, U.S.A.). All reagents for BRET^2 (a variant of BRET) were from Packard Biosciences (Pangbourne, Reading, Berks., U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.) or Fisons (Loughborough, Leics., U.K.). Oligonucleotides were from Cruachem Limited (Glasgow, Scotland, U.K.).

Construction of receptor plasmids

Production of the δ-opioid receptor–*Renilla* luciferase and β_2-adrenergic receptor–*Renilla* luciferase constructs has been described previously [16]. Thyrotropin-releasing hormone receptor-1 (TRHR-1)–enhanced yellow fluorescent protein (eYFP) was constructed from TRHR-1–enhanced green fluorescent protein (eGFP) [27] that was digested with *Hin*dIII and *Xba*I to excise the eGFP fragment. PCR amplification of the eYFP DNA fragment was achieved using pEYFP (ClonTech, Basingstoke, U.K.) as a template. The primer (5′-AGAAAGCTTATGGTGAGCAAGGGCGA-3′) included a *Hin*dIII site immediately upstream of the eYFP gene. The primer (5′-TGATCTAGATTACTTGTACAGCTCGTC-3′) was used to place an *Xba*1 site immediately downstream of the stop codon of eYFP. The substitution of eYFP for GFP was confirmed by both restriction enzyme analysis and by nucleotide sequencing. δ-Opioid receptor–GFP^2 (where GFP^2 is a mutationally modified form of GFP) was constructed using the human δ-opioid receptor in pcDNA4 as a template for PCR amplification. The primer (5′-AAAGCTAGCGCCACCATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGGAACCGGCCCCCTCCG-3′) was used to incorporate a c-myc epitope tag, a Kozak sequence and an *Nhe*1 site immediately upstream of the human δ-opioid receptor. The reverse primer (5′-ATAGGATCCGGCGGCAGCGCCAC-3′) incorporated a *Bam*H1 site immediately downstream of the sequence. The plasmid pGFPN^2 (Packard Biosciences) was digested with *Nhe*1 and *Bam*H1 to allow in-frame ligation of the human δ-opioid receptor with GFP^2. The sequence of the construct was confirmed to be correct by nucleotide sequencing. κ-opioid receptor constructs were made using rat κ-opioid receptor as a template for PCR amplification. The forward primer (5′-AAATTTGGTACCATGAAGACGATCATCGCCCTG-3′) was used to introduce a *Kpn*1 site upstream of the κ-opioid receptor gene sequence. The reverse primer (5′-GCGTCTAGCTACTGGCTTATTCATCCCACC-3′) was used to remove the stop codon and place an *Xba*1 site immediately downstream of the gene sequence. *Renilla* luciferase was PCR-amplified using the vector pRLCMV (Promega, Southampton, U.K.) as a template. The following primers were used: 5′-GCGTCTAGAACTTCGAAAGTTTATG-3′ (forward) and 5′-TCGCTCGAGTTATTGTTCATTTT-3′ (reverse). These placed *Xba*1 and *Xho*1 restriction enzyme sites upstream and downstream of the gene sequence respectively. eYFP was PCR-amplified using the vector pEYFP as a template. The following primers were used: 5′-TGATCTAGAATGGTGAGCAAGGGCGA-3′ (forward) and 5′-CTCGAGTTACTTGTACAGCTCGTC-3′ (reverse). These placed *Xba*1 and *Xho*1 restriction enzyme sites upstream and downstream of the gene sequence respectively. PCR fragments were digested appropriately and then ligated into pcDNA3.1(+) (Invitrogen, Paisley, Renfrewshire, Scotland, U.K.) to form a chimaeric construct with either *Renilla* luciferase or eYFP ligated in-frame and downstream of the κ-opioid receptor gene. Construct sequences were confirmed by nucleotide sequencing.

Cell culture and transfection

HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum and 2 mM L-glutamine. Transient transfections were performed on cells that were at 70–80% confluency with LIPOFECTAMINE™ reagent (Life Technologies) according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

Radioligand binding

Cells were grown in 10-cm dishes. Following a 48 h period post transfection, 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 buffer A [10 mM Tris/HCl (pH 7.5) and 0.1 mM EDTA] and lysed with 2×10 s bursts of a Polytron homogenizer. The homogenate was centrifuged at 500 ***g*** to remove unbroken cells and nuclei. The supernatant fraction was then centrifuged at 48000 ***g*** for 30 min and the pellet resuspended in buffer A and stored at -80 °C until use.

In single concentration assays, specific binding at the κ-opioid receptor constructs was determined by incubation of 5 nM [^{3}H]diprenorphine with membranes at 30 °C for 60 min in buffer B [50 mM Tris and 5 mM EDTA (pH 7.4)]. Comparisons of the binding characteristics of the various κ-opioid receptor constructs were performed in saturation binding studies using concentrations of [^{3}H]diprenorphine between 0.1–6.5 nM. Specific binding of [^{3}H]naltrindole (5 nM) at the δ-opioid receptor was determined by incubation with membrane preparations at 30 °C for 60 min in buffer B. Naloxone (300 μM) was used to determine non-specific binding in all cases. Specific binding of [^{3}H]dihydroalprenolol (2 nM) at the β_2-adrenoceptor was determined by incubation with membranes at 30 °C for 60 min in buffer C [(75 mM Tris, 1 mM EDTA and 12 mM $MgCl_2$ (pH 7.4)]. Propranolol (10 μM) was used to define non-specific binding.

Correlation of receptor number with fluorescence

δ-opioid receptor–GFP^2

Membrane preparations expressing this construct were serially diluted in buffer B. An arbitrary fluorescence value for each amount was then determined using a Victor^2 multi-label counter (PerkinElmer LifeSciences, Great Shelford, Cambridge, U.K.). An equivalent amount of membranes from untransfected HEK-293T cells was assayed for each point to determine background fluorescence. Excitation of GFP^2 was at 405 nm and emission from the GFP^2 determined using a 500 nm cut-off filter.

The same amounts of membrane were then used to determine receptor expression levels. In routine BRET^2 experiments, cells were counted using a haemocytometer and approx. 700000 cells/100 μl were seeded per well. Non-transfected cells were used to determine background fluorescence. Avogadro's number thus allowed the number of acceptor tagged receptors/cell to be ascertained.

κ-opioid receptor–eYFP

Membranes were prepared and receptor number correlated with arbitrary fluorescence in the manner described for the δ-opioid receptor–GFP^2 construct, except that the excitation wavelength filter was 485 nm and emission wavelength filter was 535 nm. The efficiency of transfection was monitored as described previously [16] and corrections for receptor expression per cells produced on this basis.

Correlation of receptor number with luminescence

Membrane preparations of HEK-293T cells expressing β_2-adrenoceptor–*Renilla* luciferase were diluted in PBS. To determine the luminescence in counts per second, 1.5 ml of diluted membranes were assayed in a Spex fluorolog spectrofluorimeter with the excitation lamp turned off (slit width, 10 nm; 2 s per increment), following addition of an equal volume of PBS containing 10 μM coelenterazine (Prolume). A mean value of the peak region centred at 480 nm was determined for each of the concentration points. Membranes from the same preparation were assayed for specific binding of [^{3}H]dihydroalprenolol to determine receptor expression. As approx. 3000000 cells were added to the cuvette during routine BRET experiments, it was possible to estimate the number of donor receptors present by use of Avogadro's number.

Correlation of mean fluorescence with acceptor concentration

In routine BRET experiments, transfected cells were analysed using FACS to determine the mean fluorescence per cell (mean for 10000 cells). This value was taken to represent the acceptor concentration. To convert the mean fluorescence values from FACS into receptor expression receptor per cell, the following method was adopted. Cells expressing different levels of κ-opioid receptor–eYFP were grown to the same level of confluency in 6-cm dishes. The number of cells per 100 μl was then estimated using a haemocytometer. The arbitrary fluorescence of a 100 μl sample was then measured using the Victor^2 multi-label counter, while the mean fluorescence per 10000 cells was determined using FACS. Mean fluorescence could then be directly correlated with receptor number.

BRET

Cells were harvested 48 h after transfection. Media were removed from cell-culture dishes and cells were washed twice with PBS before they were detached to form a suspension. In samples where a time course of addition of the drug was required, cells were incubated at 37 °C in PBS supplemented with 0.1 % glucose with an appropriate volume being removed at the indicated time intervals. Approx. 3000000 cells in 1.5 ml of PBS were then added to a glass cuvette. An equal volume of PBS containing 10 μM coelenterazine was added and the contents of the cuvette and mixed. The emission spectrum (400–600 nm) was then acquired using a Spex fluorolog spectrofluorimeter with the excitation lamp turned off (slit width 10 nm, 2 s per increment). For comparisons between experiments, emission spectra were normalized with the peak emission from *Renilla* luciferase in the region of 480 nm being defined as an intensity of 1.00. Energy transfer signal was calculated by measuring the area under the curve between 500 nm and 550 nm. Background was taken as the area of this region of the spectrum when examining emission of cells expressing only the donor *Renilla* luciferase.

BRET^2

Cells were washed three times in PBS and then harvested in PBS supplemented with $MgCl_2$ (0.1 g/l) and glucose (1 g/l). Cells were then counted on a haemocytometer and approx. 700000 cells dispensed per well into a 96-well, white-walled, culture plate (Packard Biosciences). DeepBlueC™ reagent (Packard Biosciences) was prepared in accordance with the manufacturer's instructions. Compounds to be tested were dissolved in the same buffer used for cell resuspension and were then dispensed into the appropriate wells of the 96-well plate to achieve a final concentration of 10 μM. The plate was left standing for 15 min at 20 °C to allow the ligands to interact with the expressed receptors. DeepBlueC™ was added to a final concentration of 10 μM, and assays were conducted immediately on either a Victor^2 multilabel counter (PerkinElmer Life Sciences) or a Fusion universal microplate analyser (Packard Biosciences) using 410 nm (bandpass 80 nm) to measure light emitted from *Renilla* luciferase/DeepBlueC™ and a 515 nm (bandpass 30 nm) filter to measure light emitted from GFP^2. The extent of energy transfer was defined as the ratio of light intensity at 515 nm to light intensity at 410 nm, with the ratio obtained from cells expressing *Renilla* luciferase alone defined as zero energy transfer.

Immunoprecipitation

GPCR co-immunoprecipitation studies were performed as described previously [16].

Confocal laser scanning microscopy

Cells were observed using a laser scanning confocal microscope (Zeiss Axiovert 100) using a Zeiss Plan-Apo 63 × 1.40 numerical aperture oil-immersion objective, pinhole of 25, and variable electronic zoom. eYFP or GFP^2 was exited using a 488 nm argon/krypton laser and detected with a 515–540 nm band-pass filter. The images were manipulated with MetaMorph software. Live cells were used for all experiments. Cells were grown on glass coverslips and mounted on the imaging chamber. Cells were maintained in Krebs–Ringer/Hepes buffer [130 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM Hepes, 1.2 mM Na_2PO_4, 10 mM glucose and 0.1 % BSA (pH 7.4)].

RESULTS

Co-expression in HEK-293 cells of forms of the human δ-opioid receptor differentially epitope-tagged at the N-terminus with the Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) and c-myc sequences can allow their co-immunoprecipitation [11,16] (Figure 1A). Such observations are consistent with constitutive homo-oligomerization of the δ-opioid receptor. Indeed, following resolution of such samples by SDS/PAGE, immunoreactive polypeptides corresponding to the δ-opioid receptor migrate in positions consistent with at least some of these oligomers being resistant to monomerization (Figure 1A). Equivalent studies with the κ-opioid receptor also resulted in the detection of constitutively formed homo-oligomeric species [11]. Furthermore, co-expression of appropriately tagged forms of δ- and κ-opioid receptors also allowed their co-immunoprecipitation [11] (Figure 1A). Co-expression of an epitope-tagged form of the δ-opioid receptor and the wild-type β_2-adrenoceptor can also result in their co-immunoprecipitation [16], and the same was observed in the present study following co-expression of a tagged form of the κ-opioid receptor with the β_2-adrenoceptor (Figure 1B).

Co-expression of GPCRs that have been C-terminally tagged with combinations of *Renilla* luciferase and eYFP can be used to

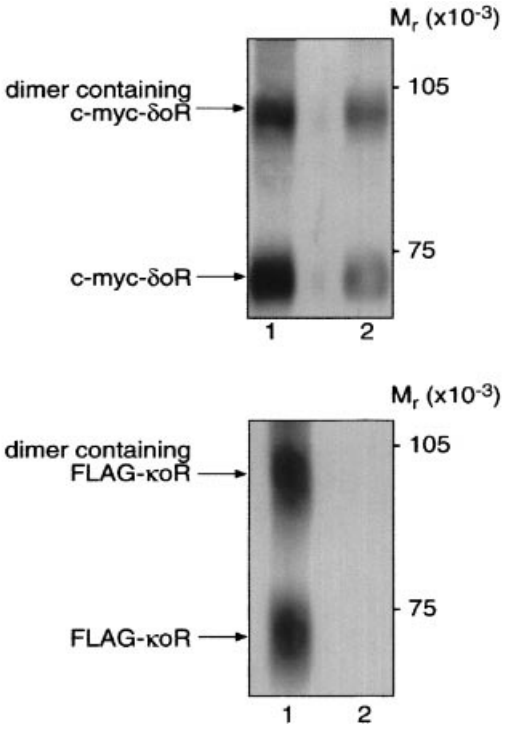

Figure 1 Co-immunoprecipitation of GPCR subtypes

(**A**) Immunoprecipitation of complexes containing opioid receptor subtypes. HEK-293 cells were transiently co-transfected with an N-terminally c-myc-tagged form of the δ-opioid receptor (c-myc-δoR; lanes 1 and 2) and N-terminally FLAG-tagged forms of either the δ-opioid receptor (lane 1) or the κ-opioid receptor (lane 2). Samples were immunoprecipitated with the M5 anti-FLAG monoclonal antibody, resolved by SDS/PAGE and immunoblotted with the A14 anti-(c-myc) antibody. (**B**) Immunoprecipitation of complexes containing both the κ-opioid receptor and the β_2-adrenoceptor. HEK-293 cells were transiently co-transfected with the β_2-adrenoceptor (lanes 1 and 2) in the absence (lane 2) or presence (lane 1) of an N-terminally FLAG-tagged form of the κ-opioid receptor (FLAG-κoR; lane 1). Samples were immunoprecipitated with an anti-(β_2-adrenoceptor) antibody, resolved by SDS/PAGE and immunoblotted with the M5 anti-FLAG antibody. M_r markers are shown on the right.

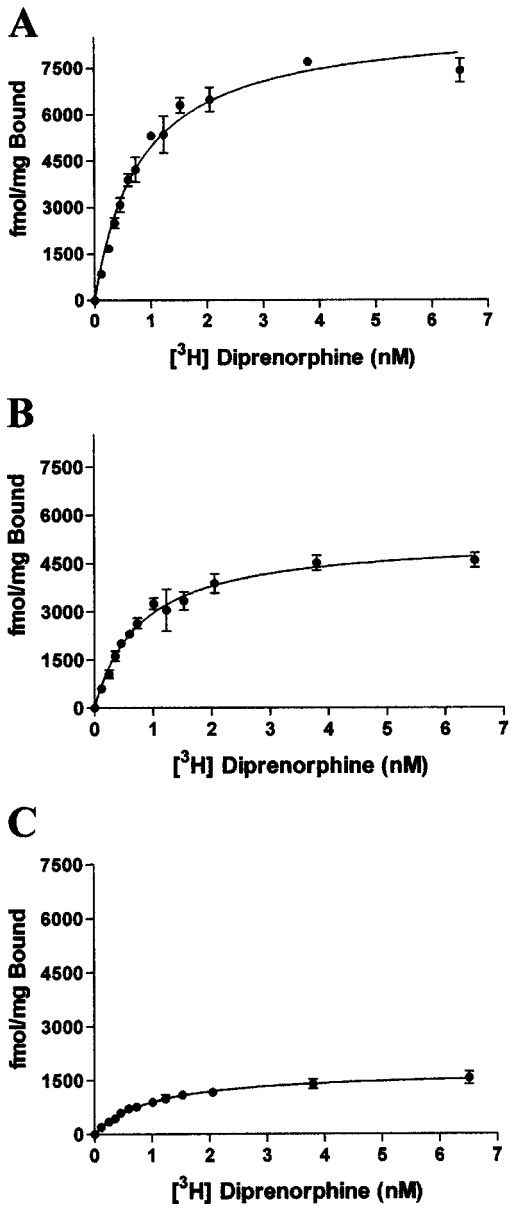

Figure 2 Expression of *Renilla* luciferase- and eYFP-tagged forms of the κ-opioid receptor

HEK-293 cells were transfected to express the κ-opioid receptor (**A**) or the κ-opioid receptor C-terminally tagged with either eYFP (**B**) or *Renilla* luciferase (**C**). Membranes were prepared and the expression of the κ-opioid receptor constructs was determined by the specific binding of various concentrations of [^{3}H]diprenorphine. Results are means $\pm$ S.E.M. from a representative experiments performed in triplicate, with similar results being obtained in three separate experiments.

examine protein–protein interactions in intact cells using BRET [14–16]. In such assays, the luciferase-tagged construct acts as the energy donor and the eYFP-tagged form as the energy acceptor. Following expression of such tagged forms of the κ-opioid receptor, membrane [^{3}H]-labelled ligand-binding studies clearly demonstrated expression of κ-opioid receptor–eYFP as an appropriately folded polypeptide capable of binding opioid ligands. Parallel expression and binding studies on the κ-opioid receptor–*Renilla* luciferase construct also demonstrated this protein to be successfully expressed. Detailed analysis of the specific binding of [^{3}H]diprenorphine to each of the isolated κ-opioid receptor and both the eYFP- and *Renilla* luciferase-tagged forms of this receptor demonstrated that the modifications at the C-terminus of the receptor did not alter substantially either the affinity to bind this ligand (Figure 2) or the ability of either the κ-opioid receptor agonist ICI199441 {2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide} or the antagonist/inverse agonist GNTI [5′-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2,3′-indolomorphinan] to compete with [^{3}H]diprenorphine for the κ-opioid receptor–ligand binding site (Table 1). Noticeably, however, in a series of studies, the *Renilla* luciferase-tagged construct was expressed at only approx. 25% of the levels of κ-opioid receptor–eYFP when cells were transfected with equal amounts of plasmid DNA encoding each form (Figure 2).

To provide a direct measure of energy acceptor expression levels for energy transfer studies, we correlated both the fluorescence intensity derived from cell membranes expressing κ-opioid receptor–eYFP with receptor expression levels measured in ^{3}H-labelled ligand-binding studies (Figure 3A) and the mean fluorescence of cells expressing this construct with receptor expression levels by use of FACS (Figure 3B). These provided a very high correlation over the range of 0–200 fmol of the receptor construct

Table 1 Ligand binding characteristics of κ-opioid receptor constructs

The affinity of [^{3}H]diprenorphine to bind the κ-opioid receptor (κ-OR) constructs was assessed in saturation binding studies. These values were used in conjunction with competition binding studies to assess the affinities of the other ligands. Results represent means ± S.E.M., $n = 3$. Luc, luciferase.

	K_d (nM)	K_i (nM)	
Construct	[^{3}H]Diprenorphine	ICI 199441	GNTI
κ-OR	0.73 ± 0.14	2.76 ± 0.06	0.30 ± 0.08
κ-OR–eYFP	0.63 ± 0.08	1.63 ± 0.21	0.10 ± 0.03
κ-OR–*Renilla* Luc	0.57 ± 0.14	1.62 ± 0.17	0.52 ± 0.13

in cell membranes (Figure 3A) and between 50000–600000 copies of the receptor per cell (Figure 3B). We also correlated expression levels of *Renilla* luciferase-tagged forms of receptors with luminescence output (Figure 3C) to provide direct measures of expression of the energy donor partner.

BRET between combinations of receptors tagged with *Renilla* luciferase and eYFP has recently been used to study homo-oligomerization of the β_2-adrenoceptor [14,16], the δ-opioid receptor [16] and the TRHR-1 [15]. Co-expression of *Renilla* luciferase- and eYFP-tagged forms of the κ-opioid receptor in HEK-293 cells also produced an energy transfer signal consistent with the presence of constitutively formed homo-oligomers of this receptor (Figure 4). Addition of neither the κ-opioid receptor agonist ICI199441 nor the antagonist/inverse agonist GNTI at near-saturating concentrations and for periods up to 30 min significantly altered the BRET signal. These ligands thus do not alter the oligomerization status of the κ-opioid receptor (Figure 4). In various studies, experiments were performed in the presence of between 30000 and 150000 copies per cell of κ-opioid receptor–*Renilla* luciferase as the energy donor. Signals that were convincingly resolved from the noise of the assay (above 10

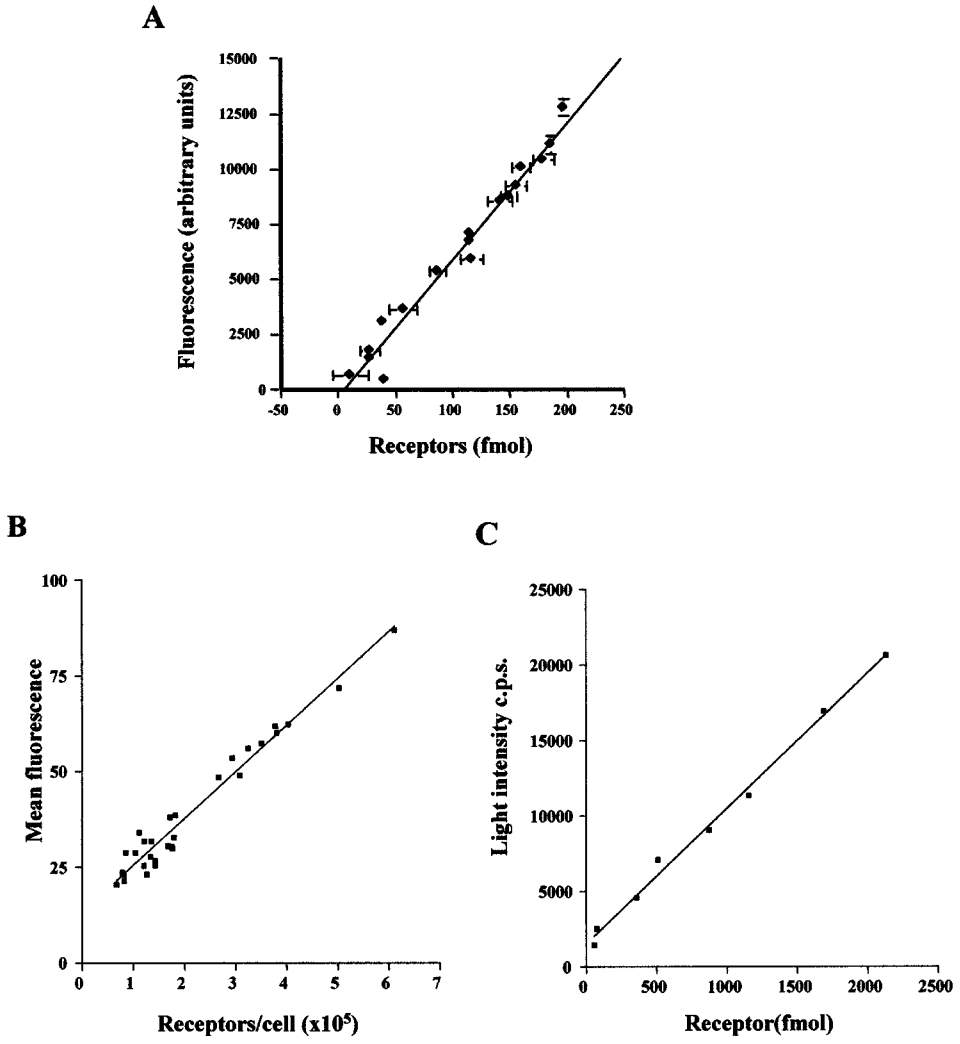

Figure 3 Correlation of the fluorescence and luminescence characteristics of receptor BRET constructs with levels of expression

(**A**) Membranes of HEK-293 cells transfected to express κ-opioid receptor–eYFP were used to measure both levels of expression of the construct by the specific binding of a near-saturating concentration of [^{3}H]diprenorphine and fluorescence intensity of the eYFP. Results are means ± S.E.M. for two experiments each performed in triplicate. (**B**) HEK-293 cells transfected to express the κ-opioid receptor–eYFP were used in FACS studies. Results are combined from three experiments. (**C**) Membranes of HEK-293 cells transfected to express κ-opioid receptor–*Renilla* luciferase were used to measure both levels of expression of the construct and luminescence output following addition of coelenterazine. Results are from a representative experiment, with similar results being obtained in three separate experiments.

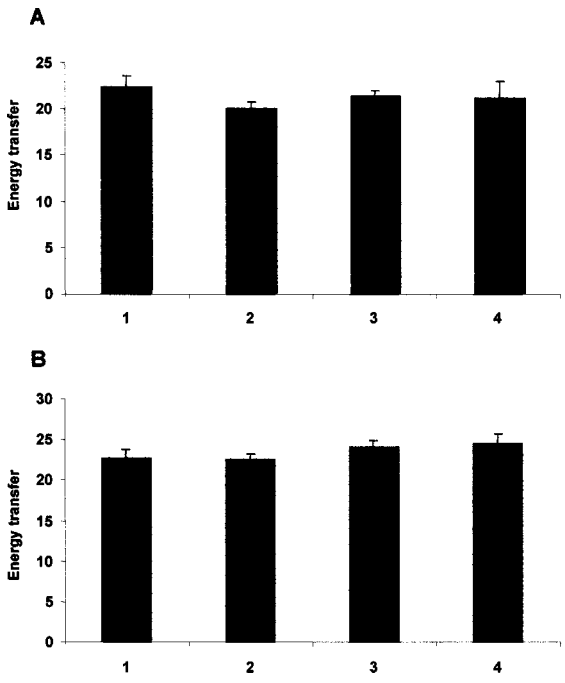

Figure 4 The κ-opioid receptor is a constitutively expressed oligomer

HEK-293 cells were co-transfected with forms of the κ-opioid receptor C-terminally tagged with either *Renilla* luciferase or eYFP. Intact cells were then used to measure BRET following addition of coelenterazine. (**A**) The κ-opioid receptor agonist ICI199441 (10 μM) was added for 0 (bar 1), 9 (bar 2), 18 (bar 3) or 28 (bar 4) min. (**B**) The κ-opioid receptor antagonist GNTI (10 μM) was added for 0 (bar 1), 9 (bar 2), 18 (bar 3) or 28 (bar 4) min. Results are means $\pm$S.E.M. from three experiments.

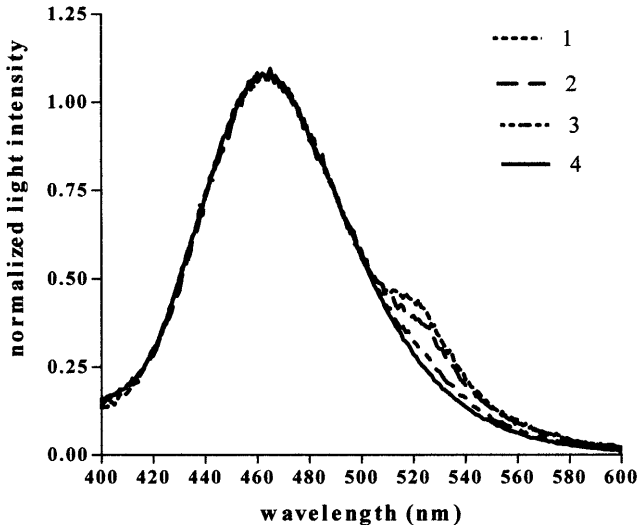

Figure 5 Sensitivity of BRET-based analysis of receptor oligomerization

κ-Opioid receptor oligomerization was assessed by co-expressing κ-opioid receptor–*Renilla* luciferase and various amounts of κ-opioid receptor–eYFP (see Figure 6 for further details). Following addition of coelenterazine to intact cells, wavelength sweeps of light emission were conducted. The energy transfer signal was calculated from the appearance of an emission shoulder centred at 527 nm above that obtained in the absence of expression of κ-opioid receptor–eYFP (see the Experimental section and [16]). Although clear signals are obtained that correspond to 34.3 (line 1) and 26.0 (line 2) energy transfer units, the data corresponding to 8 energy transfer units (line 3) is close background levels (line 4). All subsequent BRET studies thus defined an arbitrary cut off of 10 energy transfer units as the minimum accepted as being above background.

arbitrary energy transfer units) (Figure 5) required expression of approx. 75000 copies per cell of κ-opioid receptor–eYFP as the energy acceptor (Figure 6A). However, for levels of expression above 75000 copies per cell of the κ-opioid receptor–eYFP energy acceptor construct, energy transfer signals were then highly and directly correlated with levels of expression of the acceptor construct (Figure 6A).

As evidence for the capacity of κ- and δ-opioid receptors to form hetero-oligomeric complexes was obtained in co-immunoprecipitation studies (Figure 1A), we also employed BRET to explore this in intact living cells. Co-expression of δ-opioid receptor–*Renilla* luciferase and κ-opioid receptor–eYFP in HEK-293 cells also provided direct support for their capacity to generate hetero-oligomers containing both species. These were produced as efficiently as κ-opioid receptor homo-oligomers. BRET signals that were convincingly above background required a similar level of expression of κ-opioid receptor–eYFP per cell (Figure 6B) when the δ-opioid receptor–*Renilla* luciferase energy donor was expressed at similar levels as for the κ-opioid receptor–*Renilla* luciferase construct. Higher levels of expression of κ-opioid receptor–eYFP again resulted in clear energy transfer signals corresponding to the hetero-oligomer that were directly correlated with expression levels over the full range that was achieved. Indeed, the increase in energy transfer signal with acceptor expression levels corresponding to the δ–κ-opioid receptor hetero-oligomer was at least as marked as that corresponding to the κ-opioid receptor homo-oligomer (compare Figures 6A and 6B). *A priori*, it seems reasonable that more closely related GPCR sequences would be more likely to form hetero-oligomers than less closely related receptors. To test this hypothesis we co-expressed a β_2-adrenoceptor–*Renilla* luciferase construct as the energy donor and varying levels of the κ-opioid receptor–eYFP as the potential energy acceptor. It was again possible to observe an energy transfer signal. However, now approx. 250000 copies of κ-opioid receptor–eYFP per cell were required to generate a convincing energy transfer signal when the donor was present at similar levels as in the earlier studies (Figure 6C). Again, however, for expression levels above 250000 copies per cell, correlation between energy acceptor expression levels and energy transfer detection was both significant and direct. However, compared with the interactions between the opioid receptor pairs (Figures 6A and 6B), the increase in energy transfer signal with increasing levels of acceptor expression was significantly lower for the κ-opioid–β_2-adrenoceptor pairing (compare the slopes of the line in Figure 6C with those in Figures 6A and 6B). As a further test of this hypothesis, BRET studies were conducted following co-expression in HEK-293 cells of the κ-opioid receptor and TRHR-1. However, it was difficult to detect a significant energy transfer signal from this pairing over the expression levels achieved (Figure 6D).

Renilla luciferase and eYFP are a less than ideal pairing for BRET studies when luminescence produced via the oxidation of coelenterazine is used to excite the fluorescent protein, because the emission spectra of the two are poorly resolved. A positive control for BRET is provided by expression of a *Renilla* luciferase–eYFP fusion protein. Following transient expression of this construct and addition of coelenterazine, the emission corresponding to energy transfer was observed as a shoulder at 527 nm of the luciferase emission which has a peak at 480 nM (Figure 7A). Recently, a variant of BRET (BRET^2) has been introduced in which *Renilla* luciferase uses a modified form of coelenterazine, designated DeepBlueC™, as substrate. The radiant energy so produced is used to excite a mutationally modified form of GFP, designated GFP^2. We thus constructed a positive control for BRET^2 consisting of a fusion protein in which the

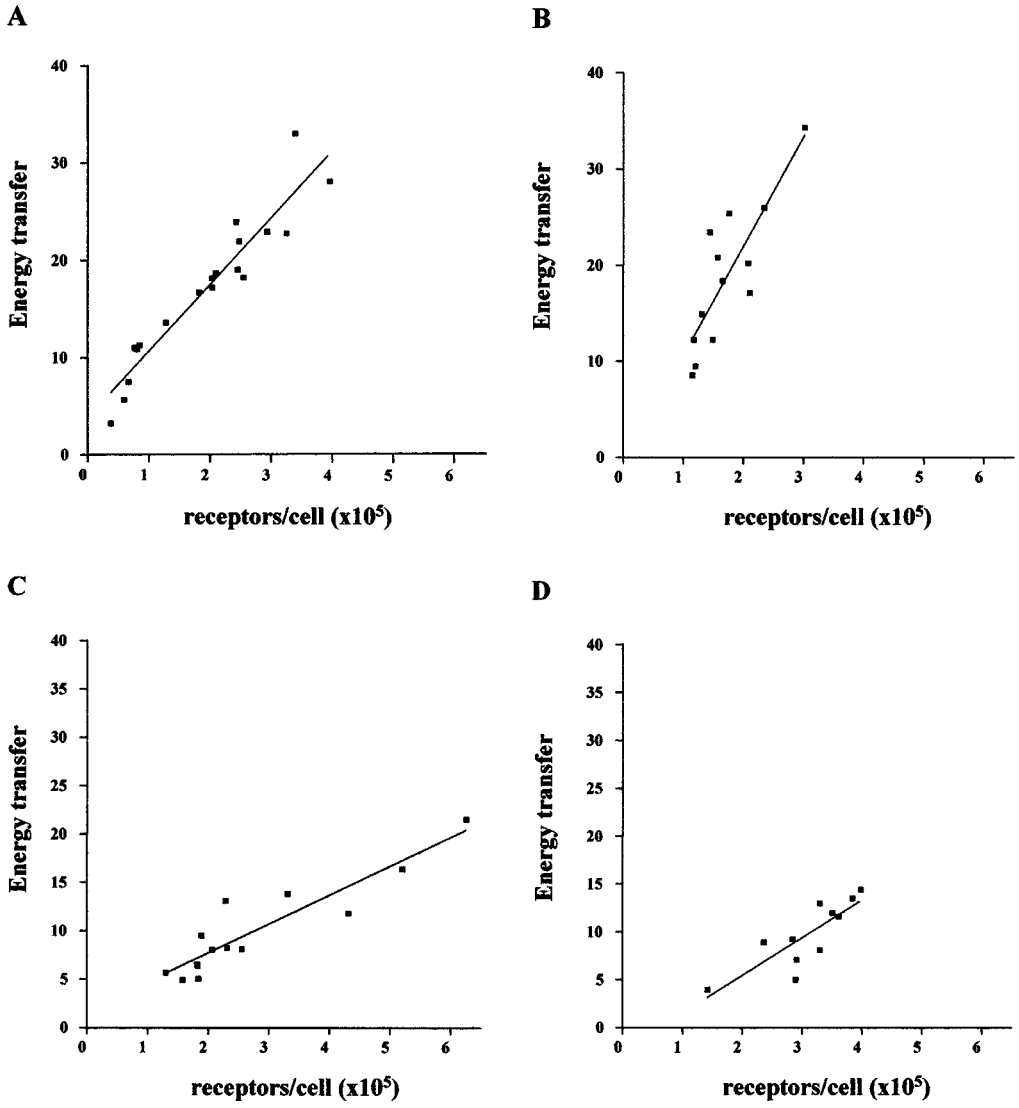

Figure 6 BRET-based detection of homo- and hetero-oligomers containing the κ-opioid receptor

Pairs of receptors that were C-terminally tagged with either *Renilla* luciferase or eYFP were expressed transiently in HEK-293 cells. BRET was measured in intact cells following addition of coelenterazine. Fluorescence of eYFP was monitored and used to determine levels of expression of the energy acceptor constructs and luminescence of the luciferase was used to monitor levels of the energy donor. Levels of expression of the eYFP-tagged forms were altered, whereas levels of the *Renilla* luciferase-tagged forms were maintained at similar levels. (**A**) Interactions between κ-opioid receptor–*Renilla* luciferase and κ-opioid receptor–eYFP. (**B**) Interactions between δ-opioid receptor–*Renilla* luciferase and κ-opioid receptor–eYFP. (**C**) Interactions between β_2-adrenoceptor–*Renilla* luciferase and κ-opioid receptor–eYFP. (**D**) Interactions between κ-opioid receptor–*Renilla* luciferase and TRHR-1–eYFP. For estimates of the expression level of the energy acceptor required to detect oligomerization, an arbitrary cut-off of 10 energy transfer units was selected. Below this level signals could not be resolved adequately from the emission spectrum of the energy donor (see Figure 5).

N-terminus of GFP² was linked directly to the C-terminal tail of *Renilla* luciferase. Transient expression of the BRET² positive control in HEK-293 cells, followed by addition of DeepBlueC™, resulted in emission of a major peak of light at 400 nm with a second smaller, but distinct and well-resolved, peak being observed at 510 nm (Figure 7B). This provides significantly improved signal to background than for the *Renilla* luciferase/eYFP pairing. A form of the δ-opioid receptor C-terminally tagged with GFP² was thus produced. As with the eYFP-tagged receptors discussed above, a strong and direct correlation between fluorescence output and construct expression levels was observed in membranes of transiently transfected HEK-293 cells (Figure 8). Fluorescence output was not significantly different between eYFP and GFP² per copy of receptor fusion expressed (compare Figures 3A and 8). δ-Opioid receptor–GFP² was then co-expressed in HEK-293 cells with the δ-opioid receptor–*Renilla* luciferase construct. Addition of DeepBlueC™ generated an energy transfer signal in which the signal to background (measured in cells expressing only δ-opioid receptor–*Renilla* luciferase) was substantially greater than recorded previously using the *Renilla* luciferase–coelenterazine–eYFP partners (Figure 9). In these experiments, the δ-opioid receptor–GFP² energy transfer acceptor was expressed at 114000 ± 5000 copies per cell (mean ± S.E.M.). However, as recorded previously for the traditional BRET partners [16], addition of neither the δ-opioid receptor agonist DADLE ([D-Ala²,D-Leu⁵]enkephalin) nor the antagonist/

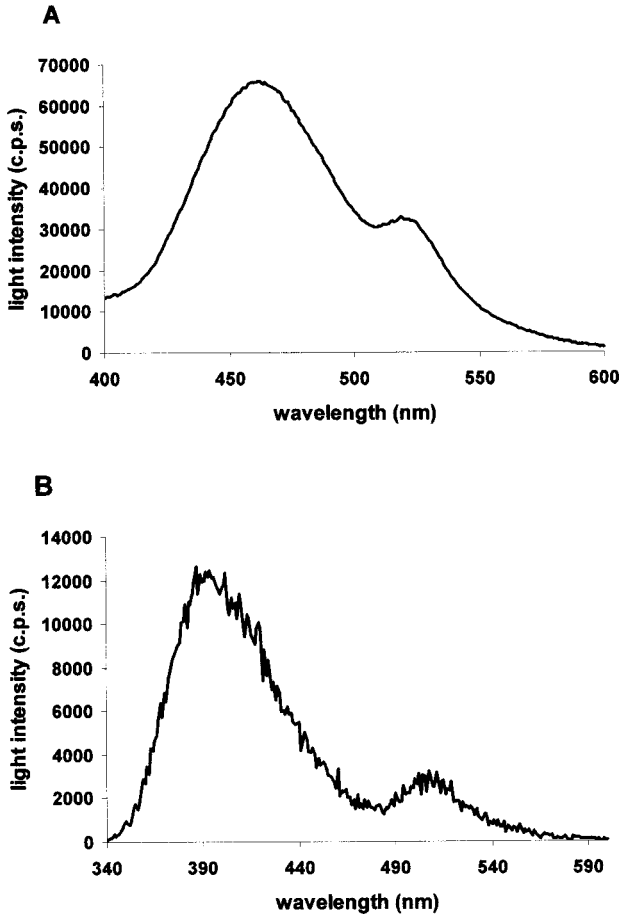

Figure 7 BRET^2 provides greater resolution of the energy transfer signal from the emission spectrum of *Renilla* luciferase than traditional BRET

Positive controls for either traditional BRET (a *Renilla* luciferase–eYFP fusion protein) (**A**) or BRET^2 (a *Renilla* luciferase–GFP^2 fusion protein) (**B**) following transient expression in HEK-293 cells. Spectra were recorded after addition of coelenterazine (**A**) or DeepBlueC^{TM} (**B**).

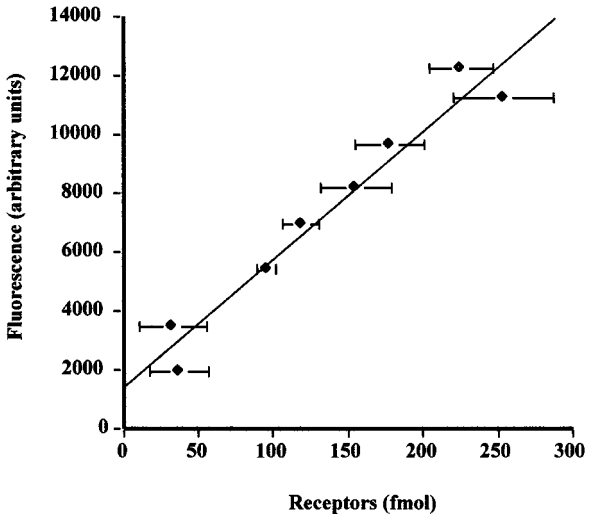

Figure 8 Correlation of the fluorescence intensity of δ-opioid receptor–GFP^2 with levels of expression

Membranes of HEK-293 cells transfected to express δ-opioid receptor–GFP^2 were used to measure both levels of expression of the construct and fluorescence intensity. Results are means ± S.E.M. from a representative experiment performed in triplicate, with similar results being obtained in three separate experiments.

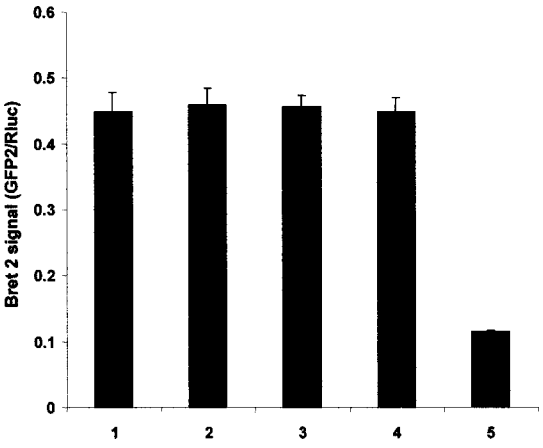

Figure 9 Detection of δ-opioid receptor oligomers using BRET^2

δ-opioid receptor–*Renilla* luciferase and δ-opioid receptor–GFP^2 were co-expressed in HEK-293 cells (bars 2–4). A positive control was provided by expression of a *Renilla* luciferase–GFP^2 fusion protein (bar 1) and a background signal by expression of only δ-opioid receptor–*Renilla* luciferase (bar 5). DeepBlueC^{TM} was added and BRET^2 signals were measured. Lack of effect of the δ-opioid receptor agonist DADLE (bar 3) and the antagonist/inverse agonist ICI174864 (bar 4) (each at 10 μM) on the BRET^2 signal was also measured in cells co-expressing δ-opioid receptor–*Renilla* luciferase and δ-opioid receptor–GFP^2. Results are expressed as the BRET^2 signal (GFP^2/Rluc, ratio of emission of GFP^2 to *Renilla* luciferase) and are means ± S.E.M. from three experiments.

inverse agonist ICI174864 (*N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu; where Aib is α-aminoisobutyric acid) altered the energy transfer signal (Figure 9). This is consistent with a lack of ability of these ligands to alter the homo-oligomerization status of the δ-opioid receptor. In studies using the *Renilla* luciferase–coelenterazine–eYFP partners, we have reported previously [16] a small degree of interaction between the δ-opioid receptor and the β_2-adrenoceptor and that these interactions were increased by the presence of an agonist for either receptor. However, these observations were produced at close to the limits of detection of the system. We thus co-expressed β_2-adrenoceptor–*Renilla* luciferase and the δ-opioid receptor–GFP^2 in HEK-293 cells and subsequently added DeepBlueC™. A clear energy transfer signal, indicative of the presence of a complex containing both the δ-opioid receptor and the β_2-adrenoceptor was observed (Table 2). However, addition of DADLE, the β-adrenoceptor agonist isoprenaline or a combination of both agonists (all at 10 μM), failed to alter the extent of the energy transfer signal (Table 2). In these experiments the δ-opioid receptor–GFP^2 energy acceptor was expressed at 152000 ± 12000 copies per cell (mean ± S.E.M.). With the distinctly improved signal-to-background ratio provided by the *Renilla* luciferase–DeepBlueC™–GFP^2 partnership, we then re-investigated the potential of hetero-oligomerization between κ- and δ-opioid receptors. Co-expression of κ-opioid receptor–*Renilla* luciferase and δ-opioid receptor–GFP^2 in HEK-293 cells, followed by addition of DeepBlueC™, produced a strong energy transfer signal. In these experiments the δ-opioid receptor–GFP^2 energy acceptor was expressed at 151000 ± 17000

Table 2 BRET^2 detection of hetero-oligomers containing both the δ-opioid receptor and the β_2-adrenoreceptor

β_2-Adrenoceptor–*Renilla* luciferase (β_2AR–*Renilla* Luc) and δ-opioid receptor–GFP^2 (δ-OR–GFP^2) were co-expressed in HEK-293 cells. BRET^2 signals were recorded in the absence of ligands or in the presence of DADLE, isoprenaline or of both agonists, following addition of DeepBlueC^{TM}. A positive control was provided by expression of a *Renilla* luciferase–GFP^2 fusion protein and a background signal by expression of only β_2-adrenoceptor–*Renilla* luciferase. Results are presented as the BRET^2 signal (GFP^2/Rluc, ratio of emission of GFP^2 to *Renilla* luciferase) and represent the means ± S.E.M. from three independent experiments.

	BRET^2 signal (GFP^2/Rluc)		
Ligand	BRET^2 positive control	β_2AR–*Renilla* Luc + δ-OR–GFP^2	β_2-AR–*Renilla* Luc
None	0.429 ± 0.003	0.273 ± 0.006	0.111 ± 0.004
DADLE (10 μM)		0.274 ± 0.008	
Isoprenaline (10 μM)		0.271 ± 0.008	
DADLE (10 μM) + isoprenaline (10 μM)		0.273 ± 0.013	

copies per cell (mean ± S.E.M.). However, as above, this signal was unaltered by addition of any combinations of agonist and/or antagonist ligands for these receptors (results not shown).

DISCUSSION

GPCRs can exist as either dimers or higher oligomers [5–7]. Evidence in favour of this scenario has been derived from a wide range of approaches that span basic pharmacological analyses of the binding characteristics of receptor ligands to biophysical studies. Although many studies have been performed following transient expression of GPCRs in heterologous systems, there are increasing numbers of studies that indicate the presence and formation of receptor oligomers in native cells and tissues [10,12,28–30]. One of the most fascinating recent developments has been the growing evidence for hetero-oligomerization between distinct GPCR gene products [6] and that such oligomers can display distinct pharmacological and functional characteristics [26,31–34]. It is difficult, however, to equate the history and development of receptor pharmacology with the view that GPCR hetero-oligomerization could represent a purely stochastic process dependent only on the levels of expression of different GPCRs in the same cell. Given that GPCRs share a common structural motif based on seven transmembrane-spanning domains [3,4] that are inherently hydrophobic in nature, it would not be surprising for different GPCRs to have a degree of mutual affinity. Indeed, recent studies [35] have indicated that co-transfection of a wide range of GPCRs can result in their co-immunoprecipitation and have led to the suggestion that the natural tendency to form such oligomers restricts the usefulness and, potentially, even the validity of this approach. Therefore a key issue is to provide useful information on the relative propensity of genetically distinct GPCRs to hetero-oligomerize, and thus to begin to consider the likely portfolio of GPCR combinations that are actually present in a cell.

Resonance energy transfer techniques [11–20] have been used to explore GPCR oligomerization, primarily because they can report protein–protein interactions in intact cells. However, there has been little analysis of the limits of sensitivity of these approaches, and thus the levels of expression of GPCRs that might be required to detect a signal. On the basis of our previous demonstration [16] that δ-opioid receptor homo-oligomerization generated a larger signal than hetero-oligomerization between the δ-opioid receptor and the β_2-adrenoceptor, we now provide a framework for such quantitative studies by examining the capacity to detect interactions between closely and less closely related GPCRs.

Initial studies used a form of BRET in which energy transfer between *Renilla* luciferase- and eYFP-tagged forms of the κ-opioid receptor report homo-oligomer formation. The signal that could be clearly separated from the background was produced with expression of approx. 75000 copies per cell of the energy acceptor (the eYFP-tagged form of the receptor). In these studies, the energy donor (the *Renilla* luciferase-tagged form of the receptor) could be expressed at lower levels. A low donor to acceptor ratio is most appropriate to obtain an acceptable signal-to-background ratio in energy transfer studies in which there is substantial overlap between the two emission spectra. This is the situation with the *Renilla* luciferase–coelenterazine–eYFP pairings. It was somewhat fortuitous that we observed routinely the levels of expression of *Renilla* luciferase-tagged GPCRs in the region of only 25% of those obtained with the eYFP-tagged forms of the same receptor when using the same amount of plasmid DNA (Figure 2 and Table 1). We have noted previously [36,37] that GFP-tagged GPCRs generally express at high levels, and we assume this is related to the stability and long half-life of GFP and its variants.

The energy transfer signal, consistent with κ-opioid receptor homo-oligomerization, increased linearly with acceptor concentration over the full range of expression levels achieved. As befits GPCRs that bind highly related ligands and share the same signal transduction cascades [38], the κ- and δ-opioid receptors are closely related. They have also been reported previously [11] to form a hetero-oligomeric complex. BRET studies were consistent with their interaction, and a significant signal was again obtained with less than 100000 copies of κ-opioid receptor–eYFP as acceptor. Indeed, analysis of BRET signals produced at a range of acceptor protein expression levels indicated that the κ–δ-opioid receptor hetero-oligomer forms at least as efficiently as the κ-opioid receptor homo-oligomer (Figure 6). A major question that has been difficult to assess is whether such interactions actually represent dimeric receptor pairings or more complex arrays containing higher numbers of receptors. Evidence in favour of dimer formation includes the molecular size of fractions of co-immunoprecipitated receptors (Figure 1) and the contribution of 'domain-swapping' models of GPCR structure [25]. However, even in such co-precipitation studies, fractions of the immunoreactive material tend to migrate with higher apparent M_r and, indeed, often fail to effectively enter the resolving phase of SDS/polyacrylamide gels. Evidence in favour of higher oligomeric arrays and structural complexes for G-protein-mediated signal transduction is derived predominantly from older studies that included target size analysis and analysis of the sedimentation characteristics of detergent solubilized samples (see [39] and references cited therein).

As interactions between the β_2-adrenoceptor and the κ-opioid receptor have been reported in co-immunoprecipitation and co-internalization studies [16,33], we also used the same BRET strategy to re-examine this. Interaction was again detected, but convincing data required the expression of approx. 250000 copies per cell of κ-opioid receptor–eYFP. As the β_2-adrenoceptor energy donor construct was expressed at least as well as the two opioid receptor–*Renilla* luciferase constructs, these data provide clear evidence that formation of an oligomer containing both of the κ-opioid receptor and the β_2-adrenoceptor is less favoured. There is no background data to indicate that oligomers containing both κ-opioid receptors and the TRHR-1 are likely to

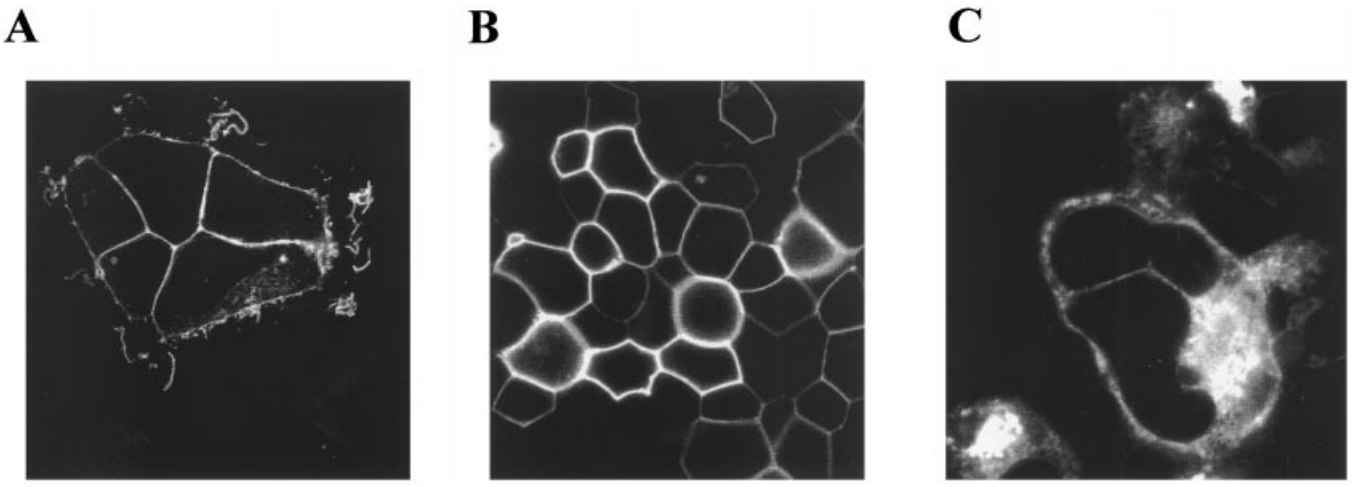

Figure 10 Distribution patterns of GFP-tagged GPCRs following transient expression in HEK-293 cells

HEK-293 cells grown on glass coverslips were transiently transfected with (**A**) κ-opioid receptor–eYFP, (**B**) TRHR-1–eYFP and (**C**) δ-opioid receptor–GFP^2 constructs. Fields of cells were then imaged as described in the Experimental section. Magnification $\times$ 472.

be of physiological significance. No clearly resolved BRET signal was detected with co-expression of this pairing containing up to 400000 copies per cell. However, although difficult to resolve from background in this region (Figure 6), the signal did appear to increase with expression levels. It is thus probable that a degree of mutual affinity between proteins of such hydrophobic nature can result in their interaction with marked overexpression [35]. It is possible that some of the growing number of reports of GPCR interactions may relate to high-level heterologous expression and not be relevant to physiological control [35].

The BRET studies discussed above are limited by the relatively poor compatability of *Renilla* luciferase and eYFP as energy transfer partners. This reflects that the emission spectrum from the luciferase tends to obscure the energy transfer output from eYFP. Recently, a modified pairing (BRET^2) has become available, which depends on the use of a chemical variant of coelenterazine and a different form of GFP. The emission spectrum of the energy transfer signal with this combination is more effectively resolved and thus a marked improvement of energy transfer signal to background can be obtained. Use of this pairing confirmed the ability of opioid receptor subtypes to form both homo- and hetero-oligomers. They also allowed a more convincing demonstration of the capacity of the β_2-adrenoceptor to be recruited into hetero-complexes with opioid receptors than we were able to obtain with the traditional BRET reagents [16]. Given that the signal-to-background issue is one of the major limitations of the traditional BRET approach, the fact that this is much improved using the BRET^2 reagents indicates that it will be possible to detect convincing signals with significantly lower levels of receptor expression. This is likely to improve confidence in the physiological relevance of GPCR oligomerization. This is an important issue given that we [16] have provided evidence previously for physical interactions between opioid receptors and the β_2-adrenoceptor. The clear implication from both our current studies and those of Salim et al. [35] is that it is possible to detect interactions between many pairs of GPCRs if they have been transfected into cells at high levels. However, it is interesting to note that a number of studies have indicated that GPCR hetero-oligomerization in not entirely non-specific and driven by Mass Action. For example, even within the small family of receptors that respond to the peptide somatostatin (SST), interactions between SST receptor (SSTR)5 and SSTR1 were observed, but not between the SSTR5 and the SSTR4 [18]. The basis for these differences remains unclear, but an understanding of contact interfaces may indicate the propensity of two GPCRs to dimerize. To date, much of the work in this area has concentrated on exploring the potential of GPCR hetero-dimerization. This now needs to be tempered with a better understanding of which GPCRs are actually co-expressed in cells and whether more physiologically relevant cell types utilize trafficking and expression patterns to define the combination of GPCRs that actually interact.

A major issue in studies of both GPCR homo- and hetero-interactions has been the capacity of ligands to modulate their presence and amount. A large number of studies have indicted that agonist ligands enhance the presence of the receptor oligomers. Such evidence has been obtained in a range of energy transfer studies [14,15,18–20] as well as co-immunoprecipitation studies. These vary from reports in which constitutive oligomerization can be detected but increased in the presence of an agonist [14,15] to those in which detection of oligomerization is largely dependent upon the addition of agonist [18–20]. In contrast with these studies, we found no evidence for regulation of GPCR oligomerization whether we were measuring homo- or hetero-interactions or on addition of either agonist or antagonist/inverse agonist ligands. The basis for these discrepancies remains obscure. There is clear evidence that the hetero-dimerization of the $GABA_B1R$ and $GABA_B2R$ polypeptides required to form a functional $GABA_BR$ occurs in the endoplasmic reticulum [40] and that the receptor matures and is transported as a dimer [23]. It is tempting to speculate that this is a specialized variant of a common process, because, in many cases, co-expression of non-functional receptor mutants can restrict delivery of wild-type receptors to the cell surface by retaining the proteins in the endoplasmic reticulum [41–43]. However, there is no evidence to date to suggest that ligand binding alters the dimerization of the $GABA_BR$. This may be a reflection that as a class C GPCR with a long N-terminal extension within which GABA binds, agonist interactions are less likely to modulate oligomerization status than for class A GPCRs in which the agonists bind within the

crevice formed by the topology of the transmembrane helices. To complicate matters further, evidence has recently been produced to indicate that agonists at the cholecystokinin receptor cause dissociation of a constitutively formed receptor complex [44].

A further, but related, issue in the use of BRET is that the signal is not restricted to receptor constructs that have been successfully delivered to the plasma membrane, even if it might be expected that only this fraction would be located appropriately to respond to ligands. Certainly, for each of the eYFP and GFP² constructs used in these studies, a significant proportion was delivered to the plasma membrane, as observed in cell-imaging studies (Figure 10). However, as is usual in such transient-expression studies, there was clearly a fraction of the expressed protein that was intracellular. Although it is not possible to directly visualize the cellular location of the *Renilla* luciferase-tagged receptors, it is clear from previous studies that these are also accessed by ligands in transiently transfected intact cells [45].

Despite these caveats, the current studies provide direct evidence for the presence of constitutively formed homo- and hetero-oligomers of opioid receptor subtypes in intact cells. They also demonstrate that there is a preference for GPCRs to form hetero-oligomers containing closely related species than between more distantly related sequences.

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