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Quantitative Assessment of β_1- and β_2-Adrenergic Receptor Homo- and Heterodimerization by Bioluminescence Resonance Energy Transfer*

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Quantitative bioluminescence resonance energy transfer (BRET) analysis was applied to the study of β_1- and β_2-adrenergic receptor homo- and heterodimerization. To assess the relative affinity between each of the protomers, BRET saturation experiments were carried out in HEK-293T cells. β_1- and β_2-adrenergic receptors were found to have similar propensity to engage in homo- and heterotropic interactions suggesting that, at equivalent expression levels of the two receptor subtypes, an equal proportion of homo- and heterodimers would form. Analysis of the data also revealed that, at equimolar expression levels of energy donor and acceptor, more than 80% of the receptor molecules exist as dimers and that this high incidence of receptor dimerization is insensitive to receptor density for expression levels varying between 1.4 and 26.9 pmol of receptor/mg of membrane protein. Taken together, these results indicate that most of the receptors expressed in cells exist as constitutive dimers and that, at least in undifferentiated fibroblasts, the proportion of homo- and heterodimers between the closely related β_1- and β_2-adrenergic receptors is determined by their relative levels of expression.

G protein-coupled receptors (GPCRs)[1] represent the largest family of transmembrane receptors involved in cell signaling. In the past few years, many studies indicated that GPCR dimerization can occur between two identical receptors (homodimerization), between two different receptor subtypes of the same family, or even between receptors that are only distantly related (heterodimerization) (for a review, see Refs. 1 and 2). In most instances, co-immunoprecipitation was used as the primary experimental evidence supporting the existence of such dimers. More recently, however, light resonance energy transfer techniques such as fluorescence and bioluminescence resonance energy transfer (FRET and BRET) were also used. These "non-invasive" proximity-based assays confirmed that GPCR dimerization does not represent biochemical artifacts due to receptor solubilization and can occur in living cells. They have been used to demonstrate homodimerization of the β_2-adrenergic (3), the yeast alpha mating factor (4), the SST5 somatostatin (5), the gonadotropin releasing hormone (6), the luteinizing hormone (7), the δ-opioid (8), the thyrotropin-releasing hormone (9), the cholecystokinin (10), and the melatonin (11) receptors as well as heterodimerization between somatostatin receptor subtypes (5), somatostatin and dopamine receptors (12), melatonin receptor subtypes (11), and opioid receptor subtypes (13).

An advantage of BRET and FRET over co-immunoprecipitation approaches lies in the more quantitative nature of the assay. However, relatively few studies exploited this quantitative potential for the study of GPCR dimerization. For the melatonin receptors, Ayoub *et al.* (11) recently used BRET competition assays to determine that the transfer of energy resulted from the formation of dimers and not of higher order oligomers. They also showed that ligand binding did not alter the dimerization state of the receptors. However, other questions that could theoretically be addressed by quantitative energy transfer analysis, such as the relative affinity of the dimer partners for each other and the relative proportion of receptors engaging in dimer formation, have not yet been addressed.

β_2- and β_1-adrenergic receptors (β_2AR and β_1AR) have previously been shown to exist as homodimers (3, 8, 14, 15). The high level of sequence identity existing in domains proposed to contribute to the dimerization interface (*i.e.* transmembrane helices) (14, 16) makes them a system of choice to study their potential heterodimerization and the relative affinity of the protomers within homo- and heterotropic complexes. Although direct *in vivo* demonstration for the co-localization of the two receptor subtypes in the same cell is still lacking, the presence of β_1 and β_2AR in the same cell types has been taken as evidence for their co-expression in transitional and mid-nodal cells of the atrio-ventricular node, nerve processes of the atrioventricular and ventricular conduction systems, as well as in vascular smooth muscle cells of the kidney (17, 18). The fact that the two receptor subtypes are also found together in a large number of tissues, including liver, lung, and fat (19), gives further support to the idea that heterodimerization could occur in native tissues and warrants investigations aiming to

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; AR, adrenergic receptor; GFP, green fluorescent protein; PBS, phosphate-buffered saline; *R*luc, *Renilla* luciferase; CYP, cyanopindolol.

assess the likelihood of such intermolecular complexes.

Here, quantitative BRET approaches were applied to the study of β_1- and β_2-adrenergic receptor dimerization in a heterologous mammalian expression system. In particular, BRET saturation experiments were carried out to estimate the relative affinity of each receptor subtype to engage into homo- and heterotropic interactions. The influence of receptor density on the proportion of receptor molecules forming dimers was also assessed. Here, we report that β_1AR and β_2AR can form homo- and heterodimers and that the two receptors have similar affinities for each other and for themselves, suggesting that heterodimers are likely to form in cells expressing both subtypes. The proportion of receptor molecules forming dimers was also found to be greater than 80% at low receptor density and was constant over a 20-fold expression range.

EXPERIMENTAL PROCEDURES

Receptor Constructs

β_1AR-GFP10—The β_1AR coding sequence without its stop codon was amplified from the pBC12BI-human β_1AR plasmid (20) using sense and antisense primers harboring unique *Sac*I and *Age*I sites. The fragment was then subcloned in-frame into the *Sac*I/*Age*I site of the blue variant GFP-sapphire vector (pGFP-N1-Sapphire, Packard Bioscience) to give the plasmid pGFP-N1-β_1AR-Sapphire. Finally, the GFP-Sapphire was replaced by a green GFP variant (GFP10) containing the following mutations: P64L, S147P, and S202P. For this purpose, an *Age*I/*Bsrg*I fragment of the GFP10 variant was subcloned into the *Age*I/*Bsrg*I site of pGFP-N1-β_1AR-Sapphire to finally yield pGFP-N1-β_1AR-GFP10.

β_2AR-GFP10—The GFP10 *Age*I/*Bsrg*I fragment was subcloned into the *Age*I/*Bsrg*I site of pGFP-N1-Hisβ_2AR-YFP (3). For simplicity, GFP10 will be referred to as GFP in the remainder of the text.

β_1AR-Rluc—The pcDNA3.1-β_1AR:6aa:h*R*luc was a generous gift from BioSignal Packard. This fusion protein contains a linker of six amino acids (YGPPGS) linking the carboxyl tail of the human β_1AR and the humanized *R*luc.

β_2AR-Rluc—The humanized *R*luc coding sequence (p*R*luc(h), Packard Bioscience) was amplified using sense and antisense primers and subcloned into the PCR Blunt II Topo vector (Invitrogen). The h*R*luc fragment was excised by digestion with *Kpn*I/*Xba*I and subcloned into the *Kpn*I/*Xba*I-digested pcDNA3.1 Zeo vector to generate the pcDNA3.1 Zeo/h*R*luc plasmid. The human Hisβ_2AR coding sequence was amplified without its stop codon using sense and antisense primers. The PCR product was subcloned into PCR Blunt II Topo Vector, then excised by double digestion with *Hin*dIII/*Kpn*I and ligated into the *Hin*dIII/*Kpn*I-digested expression vector pcDNA3.1Zeo/h*R*luc. The resulting construct encodes a six-amino acid linker (GSGTGS) between the carboxyl-terminal of the β_2AR and the humanized *R*luc sequence.

Cell Culture and Transfection

HEK-293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin, 2 mM L-glutamine (all from Wisent). For transfection experiments, cells were seeded at a density of 2×10^6 cells per 100-mm dish and cultured for 24 h. Transient transfections were then performed using the calcium phosphate precipitation protocol (21). 24 h after transfection, Dulbecco's modified Eagle's medium was replaced, and the cells were then cultured in the same medium for an additional 24 h.

Forty-height hours post-transfection, cells were washed twice with PBS, detached with PBS/EDTA and resuspended in PBS/glucose 0.1%. On a routine basis, the protein concentration of the samples was determined to control for the number of cells using the Bradford assay kit (Bio-Rad) with bovine serum albumin as a standard.

BRET Measurement

We have used a slight modification of the previously published BRET assay (3, 22). The new BRET^2 technology (BioSignal Packard) takes advantage of the spectral properties of a luciferase substrate known as Deep Blue coelenterazine (DeepBlueC, Packard Bioscience), which allows a better separation between the *Renilla* luciferase (*R*luc) and the green fluorescent protein (GFP) emission spectra. Upon the catalytic degradation of DeepBlueC, the energy donor *R*luc emits light with a peak at 400 nm that allows the excitation of the energy acceptor, GFP. Once excited, GFP then re-emits fluorescence with a peak at 510 nm if the donor and acceptor molecules are within BRET-permissive distance (<100 Å).

Following harvesting, cells were distributed in 96-well microplates (white Optiplate from Packard Bioscience) at a density of ~100,000 cells per well. DeepBlueC was added at a final concentration of 5 μM, and readings were collected using a modified Top-count apparatus (BRETCount, Packard Bioscience) that allows the sequential integration of the signals detected in the 370- to 450-nm and 500- to 530-nm windows using filters with the appropriate band pass (Chroma). The BRET signal is determined by calculating the ratio of the light emitted by the Receptor-GFP (500–530 nm) over the light emitted by the Receptor-*R*luc (370–450 nm). The values were corrected by subtracting the background signal detected when the Receptor-*R*luc constructs were expressed alone.

Fluorescence and Luminescence Measurement

Cells were distributed in 96-well microplates (white Costar plate with clear bottom) at a density of ~100,000 cells per well. The total fluorescence of cells was measured using a FluoroCount (Packard Bioscience) with an excitation filter at 400 nm, an emission filter at 510 nm, and the following parameters: gain, 1; photo multiplicator tube, 1100 V; time, 1.0 s. After the fluorescence measurement, the same cells were incubated for 10 min with Coelenterazine H (Molecular Probe) at a final concentration of 5 μM, and the total luminescence of cells was measured using a LumiCount (Packard Bioscience) with the following parameters: gain, 1; photo multiplicator tube, 700 V; time, 0.5 s. For both measurements, the mean of duplicate wells was calculated. The total fluorescence was then divided by the background determined in wells containing untransfected cells. Fluorescence was expressed in -fold over background. The background was negligible for the luminescence measurements, so they were expressed as absolute values.

Radioligand Binding Assay

Forty-eight hours after transfection, ~10,000 cells (2 μg of proteins) were incubated in a final volume of 500 μl of PBS containing 0.1% bovine serum albumin with a saturating concentration (250 pM) of the β-adrenergic antagonist [^{125}I]cyanopindolol ([^{125}I]CYP). Nonspecific binding was determined as the residual binding observed in the presence of 10 μM alprenolol (Sigma). Binding reactions were carried out at room temperature for 90 min and stopped by rapid filtration over Whatman GF/C glass-fiber filters. Receptor densities are expressed in femtomoles of receptor per milligram of total cell proteins assuming one binding site per receptor molecule. Linear regression curves between the luminescence and fluorescence signals and the number of receptor determined by radio-ligand binding were then generated from cells expressing each of the constructs individually. To determine receptor surface density, the surface of HEK-293T cells was determined by measuring the average length and width of the cells under phase-contrast microscopy.

Light Emission/Receptor Binding Correction Factor

Given that the correlations between receptor numbers and the luminescence or fluorescence levels were intrinsic characteristics of each of the constructs, comparison between receptor densities derived from the light measurements required a correcting factor. This correction was achieved using the linear regression generated for each constructs (see legend of Fig. 2) and by normalizing the light emission/receptor number as a function of the steeper slope factor obtained for both *R*luc and GFP. These corrected receptor number values were then used to generate the corrected BRET saturation curves presented in Fig. 3.

RESULTS AND DISCUSSION

Homo- and heterodimerization of the β_1 and β_2AR were investigated by quantitative BRET analysis. For this purpose, human β_1 and β_2AR cDNAs were fused at their carboxyl terminus to the energy donor *R*luc and acceptor GFP. The affinities of the fusion proteins for the antagonist cyanopindolol and the agonist isoproterenol as well as the potency of isoproterenol to stimulate adenylyl cyclase were indistinguishable from those of the wild-type receptors (data not shown).

BRET and FRET approaches have been used in several studies to assess GPCR homo- and heterodimerization (2). In most cases, little attention has been paid to the ratio of donor/acceptor molecules that was used in the assays, and thus the interpretation of the data remained rather qualitative. However, controlling this parameter is essential for proper quantitative analysis. Indeed, the level of energy transfer detected for

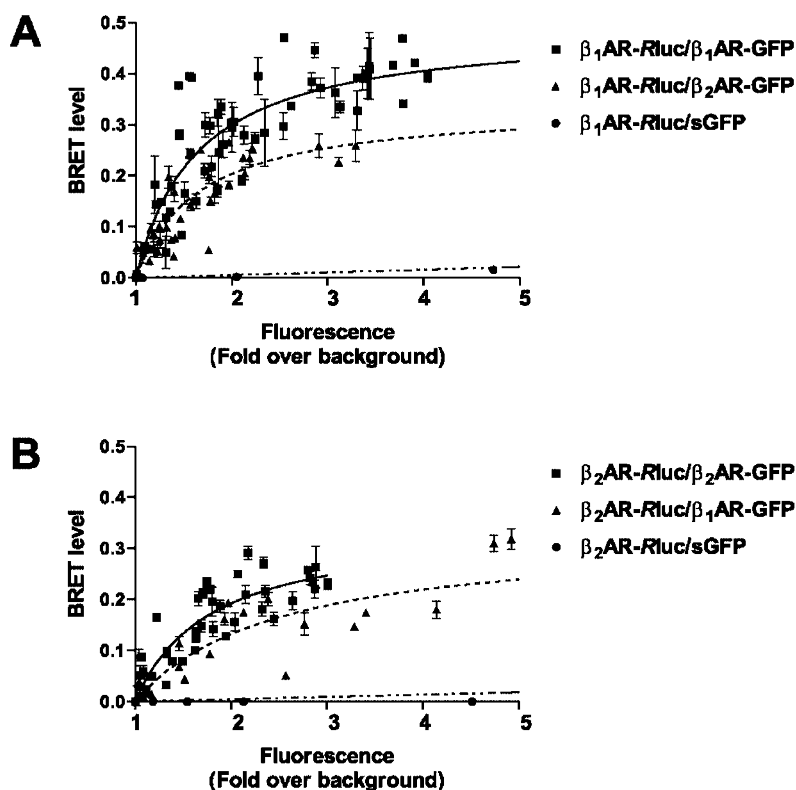

FIG. 1. **BRET saturation curves.** HEK-293T cells were co-transfected with a constant DNA concentration of β_1AR-*R*luc (*A*) or β_2AR-*R*luc (*B*) and increasing DNA concentrations of β_1AR-GFP, β_2AR-GFP, or soluble GFP. The BRET, total luminescence, and total fluorescence were measured 48 h after transfection. BRET levels are plotted as a function of the total fluorescence signal (-fold over background) used as an index for the concentration of receptor-GFP constructs expressed. The results are expressed as the mean ± S.E. of 3–10 independent experiments carried out in triplicates. The curves were fitted using a non-linear regression equation assuming a single binding site (GraphPad Prism).

a given concentration of donor should rise with increasing concentration of the acceptor until all donor molecules are engaged by an acceptor. It follows that the energy transfer should reach a plateau and that saturation curves could theoretically be constructed. The maximal level reached should be a function of the total number of dimers formed and of the distance between the donor and acceptor within the dimers, whereas the concentration of acceptor giving 50% of energy transfer ($BRET_{50}$) should be a reflection of the relative affinity of the acceptor fusion for the donor fusion proteins. Here, we applied this theoretical framework to the study of β_1 and β_2AR homo- and heterodimerization by constructing BRET saturation curves in cells co-transfected with a constant amount of receptor-*R*luc construct and increasing concentrations of the receptor-GFP plasmids. As shown in Fig. 1, significant BRET signals were observed for the β_2AR/β_2AR and β_1AR/β_1AR pairs confirming previous findings that both receptor subtypes can form homodimers (3, 14, 15). Albeit to a lower extent, co-expression of the two subtypes also led to a sizable BRET signal for the two transfer orientations (*i.e.* β_1AR-*R*luc/β_2AR-GFP and β_2AR-*R*luc/β_1AR-GFP). In all cases, BRET increased as a hyperbolic function of the concentration of the GFP fusion construct added (assessed by the fluorescence emitted upon direct excitation at 400 nm) reaching an asymptote at the highest concentrations used. Co-expression of β_2 or β_1AR-*R*luc with soluble GFP led to marginal signals that increased linearly with increasing amount of GFP added. Stimulation with the agonist isoproterenol did not promote any consistent change in the BRET saturation curves (data not shown) indicating that the dimers form constitutively and that receptor activation does not affect their oligomerization state. However, one cannot exclude the possibility that agonist stimulation could promote assembly/disassembly cycles that do not affect the steady-state proportion of receptors engaged in dimers. The modest agonist-promoted increase in BRET, previously reported for the β_2AR homodimer using $BRET^1$ (3), most likely reflected conformational changes that could not be detected using $BRET^2$. Indeed, the better resolution between the emission peaks of *R*luc and GFP with $BRET^2$ discussed under "Experimental Procedures" is not the only difference between the two generations of BRET. In particular, the quantum yield of the *R*luc/DeepBlueC coelenterazine couple is lower than that of *R*luc/coelenterazine H, and the extent of overlap between the emission spectra of *R*luc and the excitation of the GFPs is better for $BRET^2$ than $BRET^1$. These parameters can influence the sensitivity of the assays to detect small changes in distance between energy donors and acceptors. The notion that certain BRET configurations but not others allow the detection of conformational changes induced by ligands is also well exemplified by the recent observation that agonist and antagonist binding increased the BRET between the melatonin MTR1-*R*luc and MTR2-GFP but not between MTR2-*R*luc and MTR1-GFP (11).

Although the curves generated concur with the theoretical behavior predicted above, quantitative analysis is complicated by the lack of direct information provided by the fluorescence and luminescence measurements on the precise concentration of receptor molecules expressed. In the absence of such information, determining the relative affinity of the protomers for each other, based on these saturation isotherms, would require that the correlations between light emission and the number of receptor-GFP and -*R*luc fusion molecules are linear and identical for the two receptors considered. To directly test this supposition, cells were transfected with increasing concentrations of receptor-GFP and -*R*luc constructs. For each DNA concentrations, the total expression level of the receptors was determined using the lipophilic ligand $[^{125}I]$CYP, whereas the total luminescence and total fluorescence emitted by the *R*luc and GFP fusion proteins were measured following addition of the *R*luc substrate coelenterazine H and direct excitation of the GFP at 400 nm, respectively. Fig. 2 illustrates the correlation obtained between the number of total binding sites and either the luminescence or fluorescence emitted by each of the receptor fusion molecules. Even though the regression curves were highly linear, their slopes were different for the two receptors

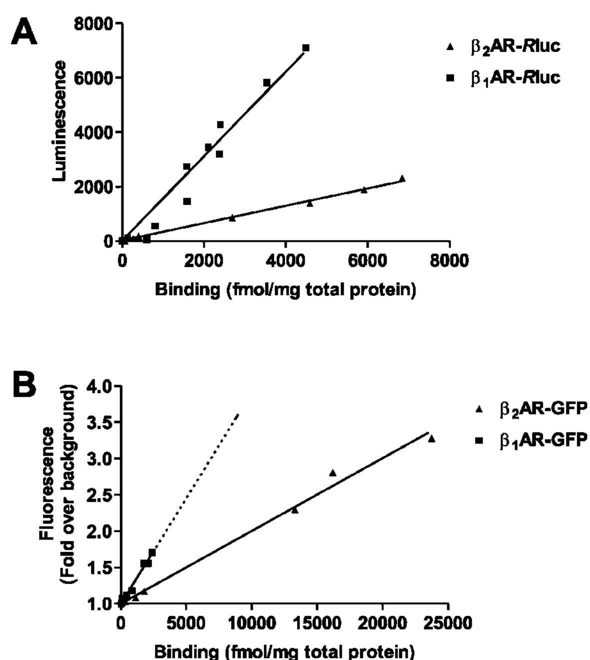

FIG. 2. **Linear relationship between total luminescence (*A*) or total fluorescence (*B*) and receptor density.** HEK-293T cells were transfected with increasing DNA concentrations of β_1 or β_2AR-*R*luc (*A*) or -GFP (*B*) fusions. Total receptor density was determined by radioligand binding assays using [^{125}I]CYP as the tracer. The total fluorescence was measured following excitation at 400 nm and detection at 510 nm, whereas the total luminescence was recorded following the addition of the *R*luc substrate, coelenterazine H. The linear regression curve was generated using GraphPad Prism. The *dotted line* corresponds to the extrapolation of the linear regression for the β_1AR-GFP. The linear regression equations used to calculate the receptor amount for a given luminescence or fluorescence intensity are as follows: β_1AR-*R*luc: y = 1.5540(x) + 20.00; β_2AR-*R*luc: y = 0.3192(x) + 20.00; β_1AR-GFP: y = 0.0002902(x) + 1.00; β_2AR-GFP: y = 0.0001004(x) +1.00.

considered. Indeed, the GFP and *R*luc signals increased more rapidly with receptor number for the β_1 than the β_2AR. Although the exact cause for this difference remains unknown, this obviously complicates the analysis of the data, because this difference must be taken into account to assess the relative affinity of the receptors for each other in BRET saturation curves. The linear regression equations derived from Fig. 2 were thus used to transform the luminescence and fluorescence value in receptor number. Although the BRET saturation curves were carried out using a fixed concentration of the *R*luc fusion partners, co-transfecting an increasing quantity of the GFP constructs introduces some levels of variability in the amount of receptor-*R*luc expressed in each case. To rule out the influence of this variable, the BRET levels were plotted as a function of the ratio between the receptor-GFP/receptor-*R*luc numbers.

As shown in Fig. 3, the BRET saturation curves generated following these corrections also behaved as hyperbolic functions reaching a saturation level. The aspect of these curves greatly contrasts with that of the curve predicted if the observed BRET resulted from random collisions promoted by a high receptor density. Indeed, a quasi-linear curve would be expected if such "bystander" BRET was taking place (23, 24). The schematic illustration of the predicted distribution of the energy donor and acceptor in the case of dimerization *versus* random collision, presented in Fig. 3*C*, allows for an intuitive appreciation of the difference between the two situations. To allow a more quantitative comparison, the progression of bystander BRET as a function of increasing concentration of the receptor-GFP in a given surface was modeled for the β_2AR homodimer. For this purpose, the receptor surface density was estimated by microscopic measurements of the HEK-293T cell surface (240 μm^2) and determining the receptor number using the equations in Fig. 2. The average receptor-*R*luc surface density in our BRET saturation experiments was found to be 3000 receptors/μm^2. Fig. 3*D* shows simulations of bystander BRET carried out for receptor-*R*luc levels of 30, 300, and 3000 receptors/μm^2. This was accomplished using a Monte-Carlo approach that assumes a random and uniform distribution of the receptors on the calculated surface. Considering that the diameter of GPCR has been estimated to be ~50 Å, assuming that, in the case of bystander BRET, receptor molecules would not be intertwined, and given that the R_0 (the distance at which the energy transfer reaches 50% of its maximum) is ~50 Å, we estimated the BRET permissive surface as $\pi \cdot 50$ Å^2. As shown in Fig. 3*D*, the predicted bystander BRET curves differed significantly from the BRET saturation curve obtained experimentally. Indeed, they progressed quasi-linearly up to receptor-GFP surface density for which experimental BRET values have already reached saturation. It should be noted that the difference between the experimental BRET saturation curve and the bystander BRET modeled for a receptor surface density of 3000/μm^2 is most likely being underestimated, because the surface measurements did not account for plasma membrane details such as microvilli that would contribute to increased cell surface area. Moreover, the number of calculated receptors reflects the total cellular receptor content and not only those present at the cell surface. The aspect of the experimental BRET saturation curve thus suggests a clustering of the energy donor and acceptor molecules resulting form receptor oligomerization rather than from their random collisions.

In an effort to distinguish between dimers and higher order oligomers, we modeled the BRET saturation curves using an equation (modified from Ref. 25) that describes the probability of forming BRET competent complexes as a function of the number of receptors within a complex (*i.e.* dimer *versus* trimer *versus* tetramer, etc). Fig. 3*D* shows that the experimental curve fits better to the theoretical dimer curve than that predicted for a trimer, suggesting that the BRET obtained from co-expression of β_2AR-*R*luc and β_2AR-GFP results from the formation of dimeric complexes of this receptor.

When comparing the BRET saturation curves obtained for the β_1 and β_2AR homo- and heterodimers (Fig. 3, *A* and *B*), similar BRET$_{50}$ values were obtained for all pairs considered (Table I), indicating that the receptors had similar relative affinities for one another. This has important implications, because it suggests that, under basal conditions, β_1 and β_2AR homo- and heterodimers have a similar probability of forming when the two receptors are heterologously expressed in HEK-293T cells. Co-localization of the two β-adrenergic receptors has been documented in numerous tissues (19, 26, 27). Although it has not been easy to experimentally demonstrate co-localization in the same cells, their expression in the same cell types has been taken as evidence for the co-existence of the two receptor subtypes (17, 18). Thus, the equal chance of forming homo- and heterodimers found in the present study could have important physiological consequences. However, future studies will be required to determine if heterodimerization can occur in native tissues.

The high likelihood of heterodimer formation between homologous receptors has also been suggested by the recent observations of Ramsay *et al.* (13) showing that homo- and heterodimerization of the δ- and κ-opioid receptors occurs at comparable levels of receptor expression. Although heterodimerization could also be observed between the distantly

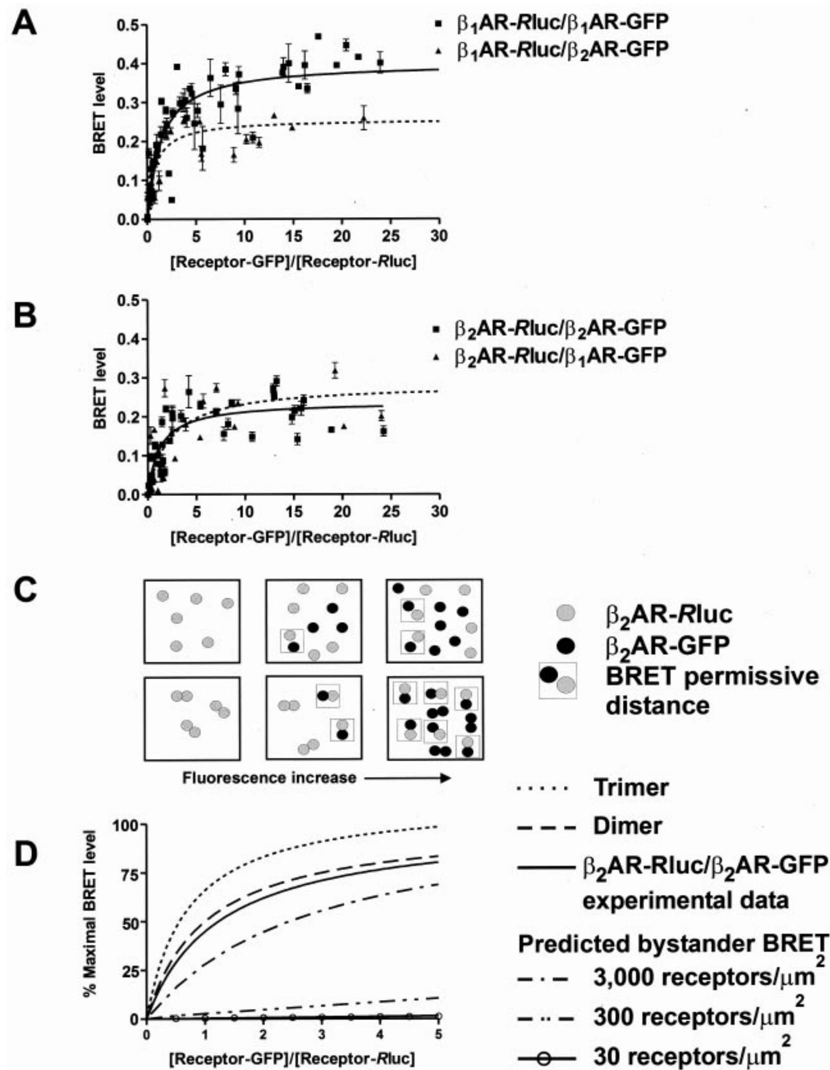

FIG. 3. *A* and *B*, corrected BRET saturation curves. The fluorescence and luminescence data from Fig. 1 were transformed into receptor numbers using equations from Fig. 2 and taking the slope factor into account as indicated under "Experimental Procedures." The BRET levels are plotted as a function of the ratio of [receptor-GFP]/[receptor-*R*luc]. The total number of receptors expressed ranged as follows: β_1AR/β_1AR, 0.8–23 pmol/mg; β_1AR/β_2AR, 0.8–17 pmol/mg; β_2AR/β_1AR, 0.3–37 pmol/mg; and β_2AR/β_2AR, 0.3–8 pmol/mg. The curves were fitted using a non-linear regression equation assuming a single binding site (GraphPad Prism). *C*, schematic representation of the distribution of the energy donor (*Receptor-Rluc*) and acceptor (*Receptor-GFP*) in the case of random collision (*upper panel*) *versus* dimerization (*lower panel*) when the density of energy donor is maintained constant while that of the acceptor is increased. *D*, modeling of the evolution of BRET resulting form random collision between energy donor and acceptor ("bystander BRET") as a function of increased acceptor surface density for donor surface densities of 30, 300, and 3000 receptors/μm^2. We simulated this process using a Monte-Carlo approach based on the assumption of random distribution. For each incremental increase of receptor-GFP, we modeled the probability P of interaction with any receptor-*R*luc as $P = 1 - (1 - p)^n$, where p is defined as the ratio of the BRET permissive surface of the receptor-*R*luc ($\pi \cdot 50 Å^2$) over the total calculated surface of the cell and n is the number of remaining receptor-*R*luc available. The experimental data of β_2AR-*R*luc/β_2AR-GFP presented in *B* are also compared with the expected BRET saturation curve for trimeric and dimeric complexes using an equation that describes the probability of forming BRET competent complexes, BRET competent complex = $(n \cdot d^{n-1} \cdot a + n \cdot d \cdot a^{n-1})/(n \cdot d^n + n \cdot d^{n-1} \cdot a + n \cdot d \cdot a^{n-1})$, where n is the number of receptor molecule in the complex, d is the number of receptor-*R*luc (energy donor), and a the number of receptor-GFP (energy acceptor). The curves expressed as percent maximal BRET are plotted as a function of the [receptor-GFP]/[receptor-*R*luc] ratio.

related opioid and β_2-adrenergic receptors (8, 28), this apparently takes place only at much higher receptor expression levels, indicating that heterodimerization is more likely to occur between receptors sharing significant sequence homology.

When considering the maximal BRET values obtained in the present study, only the β_1AR-*R*luc/β_1AR-GFP pair was found to be significantly different from the others. Indeed, the $BRET_{max}$ obtained for this pair was ~1.5 times that observed for the other combinations (Table I). As indicated above, this could indicate either that a larger proportion of β_1AR than β_2AR can engage in dimerization or that the relative position of *R*luc and GFP within the β_1AR homodimer is more permissive to energy transfer (shorter distance and/or better orientation of the dipoles). Given that the relative affinities between each of the partners were found to be very similar (Table I), the second hypothesis is more likely.

One of the major concerns when considering the physiological relevance of GPCR dimerization is the possibility that the high expression levels used in most studies could cause spurious interactions between receptors. If this were the case, one would expect that increasing the total level of receptor expression would lead to a proportional increase in dimer formation. In contrast, if dimerization can occur independently of receptor overexpression, no such relation should be expected. To distinguish between these two possibilities, BRET between β_2AR-*R*luc and β_2AR-GFP was determined for expression levels ranging from 0.44 to 46.6 pmol/mg of protein. As indicated by the saturation curves presented above (see also Ref. 23), such com-

TABLE I
Parameters obtained from BRET saturation curves

The $BRET_{max}$ is the maximal BRET obtained for a given pair and the $BRET_{50}$ corresponds to the concentration of acceptor giving 50% of the $BRET_{max}$. The results are expressed as the mean ± S.E. of 6–10 independent experiments and were derived from the data presented in Fig. 3.

	$BRET_{max}$ ± S.E.	$BRET_{50}$ ± S.E.	
β_1AR-*R*luc/β_1AR-GFP	0.40 ± 0.015	1.4 ± 0.26	*n* = 10
β_1AR-*R*luc/β_2AR-GFP	0.26 ± 0.015	0.76 ± 0.16	*n* = 7
β_2AR-*R*luc/β_1AR-GFP	0.28 ± 0.029	2.2 ± 0.77	*n* = 6
β_2AR-*R*luc/β_2AR-GFP	0.24 ± 0.015	1.2 ± 0.33	*n* = 7

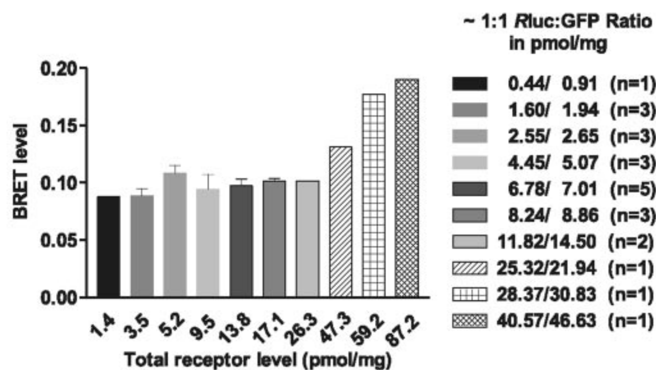

FIG. 4. **Effect of receptor expression levels on BRET determined at approximate equimolar [donor]/[acceptor] ratios.** HEK-293T cells were co-transfected with increasing quantities of β_2AR-*R*luc and β_2AR-GFP. The transfection conditions were established so that both receptor constructs were expressed at approximate equimolar levels, as determined by the total fluorescence and luminescence of the fusion proteins. BRET is expressed as a function of the total number of receptor expressed. The amounts of β_2AR-GFP and β_2AR-*R*luc are also indicated (*right part* of the *panel*). The data presented were obtained for a total of 23 independent transfections. The BRET values were then grouped according to the expression levels determined. When the data from more than two transfections were grouped, BRET values are presented as the mean ± S.E. (*n* = 3–5).

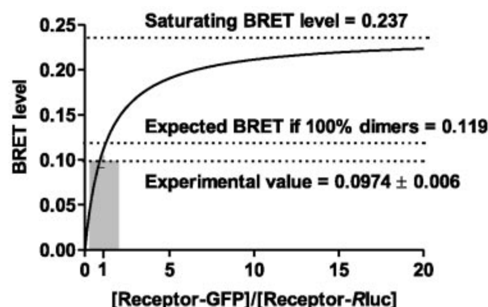

FIG. 5. **Schematic representation of the estimated percentage of β_2AR dimers in living cells.** Corrected BRET saturation curves indicated that a $BRET_{max}$ of 0.237 could be obtained for the β_2AR-*R*luc/β_2AR-GFP pair. Assuming a free equilibrium between the two constructs, only 50% of the dimers should contribute to the BRET signal when donor and acceptor are expressed at equimolar concentrations. Thus a BRET signal of 0.119 would be expected if 100% of the β_2AR existed as dimers. The average BRET value of 0.0974 experimentally observed upon expression of β_2AR-*R*luc and β_2AR-GFP at ~1:1 ratio (*n* = 20) indicates that 82 ± 10% of the total β_2AR population exist as dimers.

parison can only be carried out if the donor/acceptor ratio is maintained constant for the different expression levels tested. For this purpose, the transfection experiments were set up so as to obtain equimolar expression of the β_2AR-*R*luc and β_2AR-GFP. The expression levels were monitored by measuring the total luminescence and fluorescence signals, and the receptor number was calculated using the equations derived from Fig. 2. As shown in Fig. 4, constant BRET signals were obtained for total β_2AR levels ranging from ~1.4 to ~26 pmol/mg when equimolar concentrations of β_2AR-*R*luc and β_2AR-GFP were expressed. This indicates that a similar percentage of receptors engage in dimer formation over a 20-fold range in expression levels. The fact that the BRET signal is largely independent from receptor density also confirms that it originates from dimerization and not random collision events (23). Increases in the BRET signals were observed only when the total β_2AR was expressed at 47 pmol/mg or above. Such increases in BRET at these very high expression levels may result from random collisions between evenly dispersed donor and acceptor molecules already engaged in dimers (23), or they could represent artifactual aggregation occurring only at unusually high receptor numbers. Interestingly, 47 pmol/mg corresponds to a receptor surface density of 2.4 receptors/10,000 Å^2 representing an average distance of less than 100 Å between each receptor dimer: a distance that would be permissive to bystander BRET. These results emphasize the importance of carefully monitoring receptor expression levels in these types of studies.

The lowest level of receptor expression allowing the detection of BRET in the present study was 0.3 pmol/mg of protein (see legend of Fig. 3). Such expression level is comparable to those observed in dog heart tissue (~0.5 pmol/mg) and only 3.75-fold over those reported for human heart tissue (~0.080 pmol/mg) (29–33). Taken together, these data therefore suggest that dimerization can occur at physiologically relevant expression levels and that overexpression is not responsible for this process. Consistent with this notion, homo- and heterodimerization have been documented in native tissues for a few endogenously expressed GPCR using co-immunoprecipitation (34) or Western blot analysis (35–40). Unfortunately, the lack of adequate antibodies for co-immunoprecipitating native receptors does not allow such experiments for the endogenously expressed β_1 and β_2AR.

In addition to confirming that the BRET signal observed did not result from an artifact of overexpression, the data presented in Fig. 4 make possible some estimates of the proportion of receptors engaged in dimerization. Indeed, BRET saturation experiments presented in Fig. 3 have already indicated that the maximal level of BRET that can be obtained when all β_2AR-*R*luc are bound to a β_2AR-GFP partner is 0.237 ± 0.015 (see the reported curve in Fig. 5). Assuming a free equilibrium between the *R*luc and GFP fusion proteins, one would predict that, at equimolar concentration of the two partners, only 50% of the dimers can produce BRET (β_2AR-*R*luc/β_2AR-GFP dimer) while the other half cannot (β_2AR-*R*luc/β_2AR-*R*luc and β_2AR-GFP/β_2AR-GFP dimers). It follows that, if 100% of the receptors form dimers, the maximal BRET value observed at equimolar expression levels should be 0.1185 (*i.e.* 0.237 ÷ 2). An experimental value of 0.0974 ± 0.006 (Fig. 5) was obtained when averaging 20 BRET values recorded for equimolar expression of β_2AR-*R*luc and -GFP fusions at total expression levels varying from ~1.3 to ~26 pmol/mg. Based on these calculations, 82 ± 10% of the cellular contingent of β_2AR exist as dimers.

CONCLUSION

Our results clearly show that, in addition to forming homodimers, β_1- and β_2-adrenergic receptors form heterodimers

at nearly physiological expression levels. The similar propensity of the receptor subtypes to form homo- and heterodimers (*i.e.* the comparable $BRET_{50}$ found) could have an important impact on the β_1 and β_2AR profile in cells co-expressing the two subtypes. Indeed, relatively modest changes in the expression of one subtype should have repercussions not only on the relative proportion of the heterodimer but also on the amount of homodimers of each subtype. However, the total number of receptors engaged in dimerization should not be affected, because we found that the ratio of dimer/total receptor is independent of the expression levels and remains stable at $>80\%$ for receptor concentrations spanning a 20-fold range. Although several studies suggested that GPCR can exist as constitutive dimers (2), the relatively high proportion of dimeric receptor found in the present study is the first indication that dimers may be the predominant species under basal conditions. The currently available techniques do not make assessment possible whether constitutive homo- and heterodimerization occurs to a similar extent in cells endogenously expressing β_1 and β_2AR. However, the fact that various cardiovascular diseases are associated with changes in the relative expressions of the two subtypes in heart tissues could have important impacts on the repertoire of homo- and heterodimers expressed in a given cell (41–44). Future studies will thus be required to assess the possible functional and pathophysiological consequences of βAR homo- and heterodimerization.

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