Neuropeptide Y Y4 Receptor Homodimers Dissociate upon Agonist Stimulation

MAGNUS M. BERGLUND,1 DOUGLAS A. SCHOBER, MICHAEL A. ESTERMAN, and DONALD R. GEHLERT
Eli Lilly and Company, Lilly Research Laboratories, LCC, Indianapolis, Indiana
Received June 23, 2003; accepted August 28, 2003

ABSTRACT
The pancreatic polypeptide-fold family of peptides consists of three 36-amino acid peptides, namely neuropeptide Y (NPY), peptide YY, and pancreatic polypeptide (PP). These peptides regulate important functions, including food intake, circadian rhythms, mood, blood pressure, intestinal secretion, and gut motility, through four receptors: Y1, Y2, Y4, and Y5. Additional receptor subtypes have been proposed based on pharmacology observed in native tissues. Recent studies with other G-protein-coupled receptors have shown that homo- and heterodimerization may be important in determining receptor function and pharmacology. In the present study, the recently cloned rhesus (rh) Y4 receptor was evaluated using radioligand binding, and the pharmacological profile was found to be very similar to the human Y4 receptor. To study homo- and heterodimerization involving the Y4 receptor using bioluminescence resonance energy transfer 2 (BRET2), the carboxy termini of the rhesus Y1, Y2, Y4, and Y5 receptors were fused to Renilla luciferase, and rhY4 was also fused to green fluorescent protein. Dimerization was also studied using Western blot analysis. Using both BRET2 and Western analysis, we found that the rhY4 receptor is present at the cell surface as a homodimer. Furthermore, agonist stimulation using the Y4-selective agonists PP and 1229U91 can dissociate these dimers in a concentration-dependent manner. In contrast, rhY4 did not heterodimerize with other members of the NPY receptor family or with human opioid δ and μ receptors. Therefore, homodimerization is an important component in the regulation of the Y4 receptor.

The neuropeptide Y (NPY) family of peptides consists of NPY, peptide YY (PYY), and pancreatic polypeptide (PP). These peptides regulate many important physiological functions, such as energy homeostasis, mood, and blood pressure. Currently, there are four cloned functional G-protein-coupled receptors (GPCRs), namely Y1, Y2, Y4, and Y5, that make up the NPY receptor family (see Berglund et al., 2003a for a review) in most mammals. All of these receptor subtypes are found in the brain as well as peripheral tissues. In addition, rabbits and mice have a functional Y6 receptor, whereas in primates, this receptor subtype is not functional. NPY, PYY, and PP share a similar rank order of potencies at the Y1, Y2, and Y5 receptors: NPY ≈ PYY > PP, whereas the Y4 receptor binds PP with higher affinity than it binds NPY and PYY and is thus regarded as the PP receptor. Like PP, the Y4 receptor is mainly found in the gut (Lundell et al., 1995), but binding sites for PP and Y4 mRNA have also been found in several rat brain regions, including hypothalamus and brainstem (Berglund et al., 2003a), suggesting that PP may also have direct effects on brain function. Besides the cloned NPY receptor family, several additional receptor subtypes have been proposed based on pharmacological evaluation of various tissue preparations. However, the first draft of the human genome did not provide evidence for any additional NPY receptor subtypes. Thus, it is possible that the proposed additional receptors are derived from one or several of the already cloned receptors, alone or combined, displaying a slightly modified pharmacology in native tissues due to coexpressed proteins or post-translational modifications, compared with pharmacological profiles found in isolated systems like receptors recombiantly expressed in eukaryotic cells.

Lately, it has become evident that GPCRs can form homo- and heterodimers (Devi, 2001) and that dimer formation can be essential for and modify receptor function, as shown for the opioid receptors (Jordan and Devi, 1999). The most well known case is perhaps the GABA-B receptor, where two receptors (GABA-B1 and -B2) are needed to form a functional...
unit (Jones et al., 1998). Furthermore, opioid δ (Cvejic and Devi, 1997; Jordan and Devi, 1999; McVey et al., 2001) and the β2 adrenergic (Angers et al., 2000) receptors have been found to form homodimers as well as a heterodimer (McVey et al., 2001). Some other examples of GPCR homodimers are Bradykinin B2 (AbdAlla et al., 1999) and muscarinic M3 (Zeng and Wess, 1999) receptors. Heterodimers between bradykinin B2 and angiotensin AT1 (AbdAlla et al., 2000) have also been reported. Therefore, it is important to explore the potential for homo- and heterodimers within the NPY receptor family.

A number of methods have been used to address protein-protein interactions. One of the more recent is bioluminescence resonance energy transfer (BRET), a natural process that occurs in many organisms that emit light (Xu et al., 1999). When luciferase catalyzes the reaction coelenterazine → coelenteramide, blue light (λ = 410 nm for Renilla luciferase (RLUC) catalyzing the modified version of coelenterazine, DeepBlueC in this study) is emitted. If present in close proximity (within 100 Å; Xu et al., 1999), green fluorescent protein (GFP) can act as an acceptor for the blue photon and re-emit light in the green spectra (λ = 515 nm for GFP). When attached to proteins, GFP and RLUC can be used to investigate almost any protein-protein interaction, and several groups have employed it for studies of GPCR dimerization (see Angers et al., 2002 for a review) but also for other applications (Xu et al., 1999; Boute et al., 2001; Germain-Desprez et al., 2003).

Recently, we cloned the rhY4 receptor and utilized BRET2 to study agonist-induced β-arrestin 2 interaction in the NPY receptor family (Berglund et al., 2003b). In the present study, we explored the ability of the rhY4 receptor to form homo- and/or heterodimers with the other members of the NPY receptor family. In addition, we evaluated the effects of agonist stimulation on dimer stability.

### Materials and Methods

#### Generation of Expression Constructs

Based on the reported sequence of the rhY4 receptor (Berglund et al., 2003b), one forward primer was synthesized: rhY4f containing a HindIII site with the sequence 5′-AAGCTTGAAGCTTACCATGACACCTCTCAGCTCCTC-3′ and two reverse primers, rhY4r/KS with the sequence 5′-CCGGGCGTACCTTAAATGGGATTGGACCT-3′ and a SacI site and rhY4rNSKS (5′-CCGGGCGTACCTTAAATGGGATTGGACCT-3′) containing a KpnI and a SacI site and rhY4rNSKS (5′-CCGGGCGTACCTTAAATGGGATTGGACCT-3′) also containing a KpnI and a SacI site but lacking the stop codon to make the carboxy terminally tagged constructs. The primer pair rhY4f/KS + rhY4rNSKS and the vectors pGFP2-N2 and pM were used in a PCR reaction using genomic rhesus DNA as template. A band of 1.1 kilobase was generated and gel purified. The PCR product and vectors were digested using restriction enzymes HindIII and KpnI (10 U of each for 2 h at 37°C) and gel purified. Subsequently, the cut PCR fragment was ligated into the vector pM, and subsequently sorted into six-well plates, whereas rhY4 (WT)−expressing clones were selected based on fluorescence intensity using an inverted fluorescent microscope and subsequently sorted into six-well plates, whereas rhY4 (WT)−expressing clones were randomly picked from the plate. Subsequently, clones were tested for receptor expression using 125I-hPP (PerkinElmer Life Sciences) as radioligand, as described below.

#### Receptor Binding Studies

HEK293 cells stably expressing the rhY4 (WT) receptor and a cell line expressing the recombinant rhesus Y4-GFP receptor protein were washed once with phosphate-buffered saline (PBS) and pelleted in fresh PBS. Radioligand binding assays were conducted on isolated crude membrane homogenates as previously described (Gehlert et al., 1992) using 125I-hPP as radioligand. Nonspecific binding was defined as the amount of radioactivity remaining on the filter after incubating in the presence of 0.1 μM human (h)PP (American Peptide Co., Inc., Sunnyvale, CA). Various concentrations of peptides and peptide analogs (American Peptide Co., Inc.) or 1229U91 (Eli Lilly & Co., Indianapolis, IN) were added to the incubations to determine binding affinity. For saturation binding analysis, HEK293 cells homogenates containing the wild-type and GFP−tagged Y4 receptors were incubated with 12 different concentrations of 125I-hPP for 2 h at room temperature. The results were analyzed using the Prism software package (GraphPad Software Inc., San Diego, CA). Protein concentrations were measured using Coomassie Plus protein assay reagent (Pierce Biotechnology, Rockford, IL) using bovine serum albumin standards.

#### Agonist-Induced GTP-γS Binding

Binding of [35S]GTP-γS (PerkinElmer Life Sciences) was determined as described previously (Xu et al., 2001). Briefly, 96-well Costar plates received 50 μl of buffer, 50 μl of drug, 50 μl of [35S]GTP-γS (final concentration 100 PM), and 50 μl of cell membranes (50 μg of protein). The final concentrations of reagents in the [35S]GTP-γS-binding assays were 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM MgCl2, and 50 μM phosphonate buffer (PBS) and pelleted in fresh PBS. Radioligand binding...
0.1% bovine serum albumin. Incubations were conducted for 3 h at 25°C (steady state). A scintillation proximity assay using wheat germ agglutinin beads was used to detect [35S]GTPγS binding. Basal activity was defined as binding in the absence of agonist.

**BBR2 Studies of Coexpressed GFP- and RLUC-Tagged Receptors.** HEK293 cells of the stable cell line rhY4-GFP2 were plated out in six-well dishes (30-mm diameter; Fisher Scientific Co., Pittsburgh, PA). At about 40 to 50% confluence, the cells were transfected with 1 μg of each of the following constructs: rhY1-RLUC, rhY2-RLUC, rhY4-RLUC, rhY5-RLUC, hDELTA-RLUC, hMU-RLUC, or 250 ng of pRLUC-N2 using 3 μl of FuGENE6 diluted in 97 μl of OptiMEM (Invitrogen) according to the instructions of the manufacturer. After 72 h, the cells from each well were detached by washing with 1 ml of ice-cold PBS using a pipette tip, transferred to a 1.5-ml eppendorf tube, spun in a microcentrifuge at 5000 rpm for 2 min, and resuspended in 150 μl of PBS (about 10,000 cells/μl). Cell suspension (25 μl) was dispensed into each well of a 96-well plate (OptiPlate96, white; PerkinElmer Life Sciences). Immediately before counting the plate in a Fusion instrument (PerkinElmer Life Sciences), 25 μl of modified coelenterazine (DeepBlueC; PerkinElmer Life Sciences) diluted 1:100 in PBS was added (final concentration 5 μM). The emission from each well was counted at λ = 410 nm (RLUC optimum) and λ = 515 nm (GFP2 optimum). The BRET2 ratio for each sample was calculated as follows: \( \frac{\text{sample} \text{ at } 410 \text{ nm} - \text{baseline} \text{ at } 410 \text{ nm}}{\text{sample} \text{ at } 515 \text{ nm} - \text{baseline} \text{ at } 515 \text{ nm}} \). The baseline signal was defined as the BRET2 ratio from the stable rhY4-GFP2 cell line cotransfected with pRLUC-N2 (i.e., Renilla luciferase expressed in the cytosol). The average of four wells with untransfected HEK293 cells was used to define background (BG). Each sample was run in quadruplicate, and the ratio was calculated for each replicate. As a positive control, cells express a stable cell line expressing a cytosolic fusion protein (BBR2; PerkinElmer Life Sciences) consisting of luciferase in the amino terminus and GFP2 in the carboxy terminus was run in each experiment. Two non-NPY receptors (human opioid δ and μ) were also tagged with RLUC and tested for dimer formation with the rhY4 receptor. The levels of rhY4-GFP2 in each assay were determined by adding 25 μl of cell suspension to a black 96-well polystyrene plate (Nalge Nunc International, Naperville, IL), and the fluorescence was assessed using the Fusion instrument (excitation λ = 425 ± 20 nm, detection λ = 515 ± 30 nm).

**Effect of Agonist Stimulation on Cells Coexpressing Y4-GFP2 and Y4-RLUC.** Cells in six-well dishes were transfected as described above. These experiments were carried out 48 to 72 h post-transfection. Prior to the experiment, 1 ml of media containing hPP, 1229U91, hNPY, hPYY, or h[D-Trp32]NPY was added to the cells. The Y4 agonists hPP and 1229U91 were tested at final concentrations ranging from 1 pM to 1 μM, whereas hNPY, hPYY, or h[D-Trp32]NPY were all tested at a single 100 nM concentration. Subsequently, the cells were detached, spun at 5000 rpm, resuspended in PBS, and the luminescence and fluorescence were assayed using the Fusion instrument as described above. A time course study was also performed by adding 100 nM hPP at various times before the BRET2 ratio was assayed. The effects of concanavalin A type III (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO), an inhibitor of clathrin-mediated internalization, and cycloheximide (100 μM; Sigma-Aldrich), a protein synthesis inhibitor, were also investigated by addition to the media 30 min prior to BRET2 assay.

**Chemical Cross-Linking and Western Blot Analysis.** GFP2-tagged Y4 receptors stably expressed in HEK293 cells were grown to confluence in six-well plates. Prior to cross-linking, some cells were treated with various concentrations of hPP for 1 h at 37°C. Following agonist stimulation, the cells were washed twice with ice-cold PBS. Subsequently, the cells were resuspended in ice-cold PBS containing 2 mg/ml bis[sulfosuccinimidyl]suberate (BS2; Pierce Biotechnology) and incubated at 8°C on a rocking platform. After 1 h, the cross-linker was quenched with the addition of 50 mM Tris for 15 min. The cells were pelleted, resuspended in NuPAGE lauryl dodecyl sulfate sample loading buffer (141 mM Tris Base, 106 mM Tris HCl, 2% lauryl dodecyl sulfate, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, and 10% glycerol, pH 8.5) (Invitrogen), and heated for 5 min at 95°C. Electrophoresis was conducted using an Xcell II mini cell (Invitrogen) with 7% NuPAGE acrylamide gel electrophoresis gels (Invitrogen). Proteins were transferred onto polyvinylidene difluoride membranes using an Xcell II Blot Module (Invitrogen). The presence of GFP2-tagged rhY4 receptors was detected using a 1:4000 dilution of a mouse monoclonal GFP antibody (Roche Diagnostics) and a mouse WesternBreeze chemiluminescent kit (Invitrogen). The gel was opposed to a Hyperfilm (high-performance chemiluminescence film; Amersham Biosciences Inc., Piscataway, NJ) for 30 s. The film was developed, and images were evaluated by densitometry using the MCID Elite 6.0 software (Imaging Research, St. Catharines, ON, Canada).

**Results**

**Sequence Analyses of the rhY4 Receptor.** The rhY4 receptor was cloned using PCR with primers based on the flanking sequences of the hY4 gene, and the full-length sequence has been submitted to GenBank with accession number AY149475. The coding nucleotide sequence differs from that of the hY4 gene in 45 to 47 positions and encodes a 375-amino acid protein (i.e., the same length as the human receptor) that differs from the human amino acid sequence in 15 or 16 positions. Four polymorphic positions were found. Six clones contained the sequence variant (C70, T96, T348, and T716). One of the polymorphisms resulted in a replacement of a polar amino acid to a highly hydrophobic amino acid; Arg238 (the same amino acid as in human Y4) → leucine. This position is located at the beginning of the third intracellular loop, right after transmembrane domain 5. The impact of this replacement in the third intracellular loop was tested both in binding and in dimerization assays but did not appear to cause a change in function of the receptor (data not shown). The pharmacology and dimerization data presented in this paper are derived from the variant that is most similar to the human receptor (Arg238 = C70, T96, T348, and T716).

**Expression and Pharmacological Characterization.** The rhY4-GFP2 and rhY4 (WT) receptors expressed stably in HEK293 cells bound 125I-hPP according to a saturable one-site model with dissociation constants (Kd) of 65 ± 2.6 and 31 ± 1.7 pM, respectively, and Bmax values of 17709 ± 326 and 1593 ± 31 fmol/mg protein, respectively. A very high rhY4-GFP2-expressing cell line was selected to maximize the fluorescent signal for subsequent BRET2 studies. Eight different peptides were tested for inhibition of 125I-hPP binding at the rhY4-GFP2 and rhY4 (WT) receptor constructs, and the results are presented in Table 1. In brief, the peptides bound to the rhY4 (WT) receptor with affinities and a relative rank order of potencies indistinguishable from that of the cloned hY4 receptor (Lundell et al., 1995). The correlation (r2) between the GFP2-tagged receptor to the wild type was 0.97 (Fig. 1), but the affinities were slightly lower than the rhY4 (WT) receptor. The difference was smaller for the endogenous high-affinity agonist hPP about two-fold in agreement with the Kd values seen for the radioligand. Functional coupling to G-proteins for the rhY4 (WT) receptor stably expressed in HEK293 cells was confirmed by [35S]GTPγS binding. Human PP increased binding in a concentration-dependent manner with a pEC50 value of 8.01 ±
0.16 nM (n = 3; Fig. 2). HEK293 cells expressing the rhY4-GFP² chimeric protein were subjected to the same conditions but no agonist effect on [35S]GTPγS binding could be monitored in these cells.

**BRET² Studies of Coexpressed GFP²- and RLUC-Tagged Receptors.** The BRET² ratios were assayed for the cloned rhY4 receptor coexpressed with the rhesus Y1, Y2, and Y5 receptors as well as the human δ- and μ-opioid receptors. The expression of the GFP²-tagged rhY4 receptor (as determined by GFP² fluorescence) remained the same no matter which RLUC-tagged construct was coexpressed with it. The expression levels of each of the RLUC-tagged receptors were assayed by chemiluminescence. The Y2, Y4, δ, and μ receptors tagged with RLUC achieved equivalent expression levels, whereas the RLUC-tagged Y1 and Y5 receptors were routinely expressed at levels two- to three-fold lower than the Y2, Y4, δ, and μ receptors. The Y1, Y2, and Y5 receptor subtypes showed similar BRET² ratios as the less related opioid δ and μ receptors of 0.035 to 0.065, whereas coexpression of rhY4-GFP² and rhY4-RLUC gave a value of 0.169 ± 0.009 (Fig. 3). As a positive control, BRET²+, a cytosolic fusion protein with RLUC in the N terminus and GFP² in the C terminus, was used. Homogenate from a cell line stably expressing BRET²+ gave a ratio of 0.50 ± 0.01 (n = 9).

**Effect of Agonist Stimulation on Cells Expressing rhY4-GFP² and rhY4-RLUC.** To assess the effects of agonist stimulation, cells expressing rhY4-GFP² and rhY4-RLUC were incubated in the presence of the Y4 agonists hPP and 1229U91. The addition of hPP into the growth media produced a decrease in the BRET² ratio. A time course study revealed that the maximal decrease was reached within 15 min after addition of hPP at a final concentration of 100 nM (Fig. 4a). Both hPP and 1229U91 lowered the BRET² ratio in a concentration-dependent fashion (Fig. 5), with EC50 values of 2.9 and 14 nM, respectively. In these experiments, the top BRET² ratio for hPP was 0.167 ± 0.010 and the bottom was 0.077 ± 0.005 (n = 9), whereas the corresponding values for 1229U91 were 0.163 ± 0.016 and 0.072 and 0.008 (n = 6), respectively. In contrast, no inhibition was detected when 100 nM hNPY, hPYY, or the Y5-selective agonist h[D-Trp⁴⁰³NPY was added to the growth media (Fig. 5). Concanavalin A, an inhibitor of clathrin-mediated internalization, and cycloheximide, a protein synthesis inhibitor, did not affect the agonist-induced reduction in BRET² ratio (Fig. 4b).

With both inhibitors, the reduction in BRET² ratio was similar to that observed with the nontreated cells (45–49% reduction). A small increase in both the unstimulated and agonist-stimulated BRET² ratios was observed with cycloheximide pretreatment and a decrease with concanavalin A pretreatment compared with untreated cells (Fig. 4b, columns 1 and 2), possibly due to cell toxicity.

**TABLE 1**

Peptide analogs competing against ¹²⁵I-hPP binding to HEK293 cells stably expressing the wild-type and GFP²-tagged rhY4 receptor

<table>
<thead>
<tr>
<th>Peptide and Peptide Analogs</th>
<th>rhY4 (WT) Receptor</th>
<th>rhY4-GFP² Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human neuropeptide Y</td>
<td>0.323 ± 0.037</td>
<td>3.01 ± 0.26</td>
</tr>
<tr>
<td>Human peptide YY</td>
<td>0.202 ± 0.018</td>
<td>1.32 ± 0.21</td>
</tr>
<tr>
<td>Human [d-Trp³²]NPY</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human [Leu¹¹,Pro⁶⁴]NPY</td>
<td>0.261 ± 0.043</td>
<td>1.93 ± 0.26</td>
</tr>
<tr>
<td>Human NPY (13–36)</td>
<td>15.10 ± 1.78</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human NPY (13–38)</td>
<td>11.72 ± 1.43</td>
<td>124.1 ± 4.61</td>
</tr>
<tr>
<td>Human pancreatic polypeptide</td>
<td>0.0243 ± 0.002</td>
<td>0.046 ± 0.011</td>
</tr>
</tbody>
</table>

Eleven-point displacement curves were used to determine the affinity of various peptides or peptide analogs when competing with ¹²⁵I-hPP binding to the wild-type or GFP²-tagged rhY4 receptor. The data were best fit to a one-site model, and Kᵢ values (nM) for each inhibitor were determined using Prism software. Each value represents an average of four independent determinations performed in quadruplicate (mean ± S.E.M.).
Western Blot Analysis of rhY4-GFP2 Receptors. Rhesus Y4 receptors tagged with GFP2 were chemically cross-linked using the membrane-impermeable cross-linker, BS3. Subsequent analysis of the unstimulated BS3-treated cells by nonreducing SDS-polyacrylamide gel electrophoresis and Western blotting using a GFP-specific antibody revealed two large molecular weight protein complexes, 80- and 160-kDa with relative optical densities of 0.93 and 0.86, respectively (Fig. 6, lane 1). The 80-kDa complex was the only band detected when the cross-linking reagent was omitted (Fig. 6, lane 2). The addition of 1 μM hPP produced almost a 2-fold decrease in the 160-kDa band (relative optical density 0.54) (Fig. 6, lane 4).

**Discussion**

Our group has recently cloned the Y1, Y2, Y5 (Gehlert et al., 2001), and Y4 (Berglund et al., 2003) receptors from the rhesus monkey. In this paper, we discuss the detailed characterization of the rhY4 receptor with regards to pharmacology and dimerization. In agreement with what is known about the Y4 receptor in other mammals (Eriksson et al., 1998), the rhY4 receptor has evolved much more rapidly than the other NPY receptors as the protein sequence differed from the hY4 receptor in 15 positions compared with 2, 4, and 2 for the Y1, Y2, and Y5 receptors, respectively. Four positions of the rhY4 gene were found to be polymorphic. One of the polymorphisms resulted in an amino acid replacement (Arg239/Leu239), located in the third intracellular loop, whereas the other three were silent. Interestingly, the hY4 gene has also been found to be highly polymorphic, as no less than seven positions in the coding region can differ between individuals (GenBank accession number XM_011916).

The pharmacology of the rhY4 receptor (Arg239) was found to be indistinguishable from that of the hY4 receptor (Bard et al., 1995; Lundell et al., 1995), suggesting similar ligand recognition domains. The wild-type rhY4 receptor was also found to couple to
Dimerization of the Rhesus Y4 Receptor

G-protein (Fig. 2), demonstrating that the receptor is functional when stably expressed in HEK293 cells. On the other hand, the GFP2-tagged rhY4 receptor bound 125I-hPP with slightly lower affinity than the wild-type receptor, and there was some reduction in potency for the peptide inhibitors tested. Nevertheless, these peptides had the same rank order of potencies as observed with the native receptor, suggesting that the presence of GFP2 at the C terminus of the receptor may affect agonist affinity, but the pharmacological properties remain intact. However, the lack of agonist effect on 35S(GTPγS) at the rhY4-GFP2-expressing cells suggests that the G-protein coupling is impaired in these cells. One possibility is that the presence of the 239-amino acid GFP2 molecule attached to the C terminus of the receptor affects coupling to G-proteins and thus shifts the equilibrium of the receptor-pool toward more receptors being in low-affinity conformation with regards to agonist binding and with a low ability to couple to G-proteins. Similarly, the very high expression of the rhY4-GFP2 receptor may lead to a high receptor-to-G-protein ratio, which may also result in uncoupled receptors.

Some of the first convincing pieces of evidence for dimerization or oligomerization of GPCRs came from studies using “domain swapping” between receptors (Maggio et al., 1995). Dimers between receptors of most of the major GPCR families have now been found (Gomes et al., 2001). Thus, it appears that dimerization of GPCRs is a general and important feature among these receptors. In the present study, we evaluated the ability of the rhY4 receptor to form dimers using BRET2 technology. The main advantage of BRET2 over its predecessor is the larger gap between the optimal wavelength for the luminescence from DeepBlueC and fluorescence from GFP2 (105 nm) compared with earlier versions of BRET. High BRET2 ratios, suggesting the close proximity (i.e., receptor dimerization) of GFP2 and RLUC-tagged rhY4 receptors, were observed.

This homodimerization was confirmed by cross-linking and subsequent Western blot analysis. In these studies, dimer formation was demonstrated by the detection of a high-molecular weight band of 160 kDa after incubating with cross-linking reagent BS3. Previously, the molecular weight of the hY4 receptor expressed in Chinese hamster ovary cells has been shown to be 60 kDa (Voisin et al., 2000). Based on the molecular weights we observed on the gel, the 80-kDa protein probably represents a glycosylated Y4-GFP2 monomer, whereas the 160-kDa protein is a homodimer. Similar to what has been shown for the δ opioid homodimer (Cvejev and Devi, 1997), very little of the 160-kDa band was seen without cross-linking (Fig. 6). The fact that the integrity of the Y4 homodimer does not withstand the nonreducing gel conditions suggests that covalent disulfide bonds are not involved in Y4 receptor dimerization, contrary to what is known for the metabotropic glutamate receptor 5 and the muscarinic acetylcholine receptor M3 homodimers (Zeng and Wess, 1999; Romano et al., 2001), indicating that the biochemical properties between different GPCR homodimers can vary extensively.

The rhY4 receptor did not form heterodimers with any of the other members of the NPY receptor family or with the human δ- and μ-opioid receptors (Fig. 3). However, using fluorescence resonance energy transfer and fluorescence microscopy, it was very recently shown that the Y1, Y2, and Y5 receptors, when expressed individually in baby hamster kidney cells, form homodimers (Dinger et al., 2003). In the present study, the corrected BRET2 ratio for the rhY4 homodimer (0.17) was almost three times higher than that found when rhY4-GFP2 was coexpressed with rhY2-RLUC. Still, all receptors (Y1, Y2, Y5, δ, and μ) generated a signal that was significantly above zero (0.035–0.064; Fig. 3) possibly representing a baseline for membrane-bound proteins as the baseline in these studies was defined by rhY4-GFP2 coexpressed with cytosolic RLUC.

The most common critique against the concept of GPCRs forming dimers is that overexpression of a recombinant protein in a cell line may force the receptors together in a non-natural fashion (Devi, 2001). In the present study, the cell line used for the BRET2 studies displayed a very high expression level of the rhY4-GFP2. However, the expression levels of the RLUC-tagged human opioid δ and μ receptors as well as the rhY2 receptor did not differ significantly from that of the rhY4 receptor, whereas the rhY1 and rhY5 receptors displayed two- to three-fold lower expression. Yet, the BRET2 signals of these receptors were less than a third of that of the unstimulated rhY4 homodimer, providing strong evidence that this is a specific feature of the Y4 receptor and not an artifact provoked by overexpression. However, it is possible that the high expression level might account for the high basal BRET2 signal seen for rhY4 together with the other receptors. Interestingly, the Y2 receptor had, after Y4 itself, the highest level of interaction with Y4. It has been proposed that the higher the sequence identity, the higher likelihood for dimerization (Ramsay et al., 2002). However, of the NPY receptor family, Y2 shows the lowest sequence identity to Y4, whereas the Y1 receptor is highly similar to the Y4 receptor and displayed the lowest BRET2 ratio for heterodimerization with the Y4 receptor.

In contrast to the agonist-induced homodimers observed with the β2-adrenergic (Angers et al., 2000), bradykinin B2 (AbdAlla et al., 1999), thyrotropin-releasing hormone (Kroeger et al.,...
2001; Zhu et al., 2002), and gonadotropin-releasing hormone (Kroeger et al., 2001) receptors, the rhY4 receptor complex dissociated in a concentration-dependent fashion when stimulated by the endogenous agonist, hPP, or by the bridged anti-paraldehyde dipeptide compound 1229U91 (Daniels et al., 1995; Schober et al., 1998). The symmetric structure of 1229U91 and its high affinity for Y1 and Y4 receptors has sometimes been used as an argument favoring a dimeric structure for the receptors (Daniels et al., 1995; Dinger et al., 2003). PP was more potent than 1229U91 in lowering the dimer/monomer ratio, in agreement with their affinity for the receptor (Fig. 5; Table 1). 1229U91 has been suggested to be a partial agonist at Y4 receptors. Studies of agonist induced β-arrestin 2 translocation at the rhY4 receptor showed that the maximal response of hPP was 30% higher than that from 1229U91 (Berglund et al., 2003b). However, in the present study, the maximum and minimum levels were indistinguishable, consistent with full agonism for both peptides. In contrast, a 100 nM concentration of the low-affinity Y4 agonists hNPy and hPYY or the selective Y5 agonist h[Trp^2]NPY did not affect dimer formation. Therefore, agonist-induced dissociation of the dimer is a receptor-mediated response and not a non specific action of these peptides. The agonist effect on dimer formation remained after incubation with concanavalin A or cycloheximide. These compounds are used to block clathrin-mediated receptor internalization and protein synthesis, respectively. Thus, the effect of PP on Y4 receptor dimerization may be independent of internalization. Previously, agonist-induced dimer dissociation has been shown for the δ-opioid receptor (Cvejic and Devi, 1997) and for the cholecystokinin-A receptor (Cheng and Miller, 2001). In agreement with the results from the BRET2 studies, Western blot with antibodies directed against GFP revealed that the 160-kDa protein (dimer) decreases after a 1-h stimulation by hPP (Fig. 6). It has been suggested that a change in BRET signal may merely reflect conformational changes in the RLUC- and GFP-tagged receptors rather than dimer formation (Devi, 2001). This is not the case in the present study, because a change in Y4 receptor dimer/monomer could also be detected by Western blot analysis (Fig. 6). In conclusion, we have used BRET2 and Western blot to show that the rhY4 receptor exists at the cell surface as a dimer that dissociates upon agonist stimulation. This suggests that homodimerization is important for the activation and/or down-regulation of this receptor.

References

AbdAlla S, Lother H, and Quitterer U (2000) AT1-receptor heterodimers show agreement with their affinity for the receptor (Fig. 5; Table 1). 1126 Berglund et al.

References

AbdAlla S, Lother H, and Quitterer U (2000) AT1-receptor heterodimers show agreement with their affinity for the receptor (Fig. 5; Table 1). 1126 Berglund et al.

References

AbdAlla S, Lother H, and Quitterer U (2000) AT1-receptor heterodimers show agreement with their affinity for the receptor (Fig. 5; Table 1). 1126 Berglund et al.

References

AbdAlla S, Lother H, and Quitterer U (2000) AT1-receptor heterodimers show agreement with their affinity for the receptor (Fig. 5; Table 1). 1126 Berglund et al.

References

AbdAlla S, Lother H, and Quitterer U (2000) AT1-receptor heterodimers show agreement with their affinity for the receptor (Fig. 5; Table 1). 1126 Berglund et al.