Neuropeptide Y Y4 Receptor Homodimers Dissociate upon Agonist Stimulation

MAGNUS M. BERGLUND¹, DOUGLAS A. SCHOFER, MICHAIL A. ESTERMAN,
AND DONALD R. GEHLERT

Eli Lilly and company, Lilly Research Laboratories, LCC, Indianapolis, IN 46285
Running title: Dimerization of the rhesus Y4 receptor

To whom correspondence should be sent: Donald R. Gehlert

Eli Lilly and company, Lilly Research Laboratories, LCC, Indianapolis, IN 46285
Phone: + 317 276 1840
Fax: + 317 276 5546
E-mail: gehlert_donald_r@lilly.com

Number of text pages: 21
Number of tables: 1
Number of figures: 6
Number of references: 37
Number of words in abstract: 239
Number of words in introduction: 671
Number of words in discussion: 1493

Abbreviations: PP, pancreatic polypeptide; NPY, neuropeptide Y; PYY, peptide YY;
GPCR, G-protein coupled receptor; BRET, bioluminescence resonance
energy transfer; RLUC, Renilla luciferase; GFP, green fluorescent
protein; HEK, human embryonic kidney; rh, rhesus monkey; h, human.

Section: Cellular and Molecular
ABSTRACT

The PP-fold family of peptides consists of three 36 amino acid peptides, namely neuropeptide Y (NPY), peptide YY (PYY) 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 (RLUC) and rhY4 was also fused to green florescent 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 nor 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 review) in most mammals. All 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 while 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 family receptors, 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 co-expressed proteins or post-translational modifications, compared to pharmacological profiles found in isolated systems like receptors recombinantly 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) as well as somatostatin SSTR5 and dopamine D2 receptors (Rocheville 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™ used 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 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 BRET\(^2\) 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 recently published sequence of the rhY4 receptor (Berglund et al., 2003b), one forward primer was synthesized: rhY4fH containing a HindIII site with the sequence: 5’-AAGCTTAAGCTTACCATGAACACCTCCTCACCTCCT-3’, and two reverse primers: rhY4rKS with the sequence: 5’-CCGCGGTACCTTAAATGGGATTGGACCT-3’ containing a KpnI and a SacI site and rhY4rNSKS (5’-CCGCGGTACCAATGGGATTGGACCTGC-3’) also containing a KpnI and a SacI site but lacking the stop codon in order to make the carboxy-terminally tagged constructs. The primer pair rhY4fH + rhY4rKS were used in a PCR reaction using genomic rhesus DNA as template. A band of 1.1 kb was generated and gel purified. The PCR product and the vectors pGFP2-C3, pRLUC-C1 (PekinElmer Life Sciences), and pcDNA3.1(+) (Invitrogen) were cut using restriction enzymes HindIII and KpnI (10 U of each for 2h at 37°C) and gel purified. Subsequently, the cut PCR fragment was ligated into the three different vectors cut as described above to generate the constructs GFP2-rhY4, Rluc-rhY4 and wild type (WT) rhY4. The primer pair rhY4fH + rhY4rNSKS and the vectors pGFP2-N2 and pRLUC-N2 were treated similarly to generate the carboxy-terminally tagged constructs rhY4-GFP2 and rhY4-RLUC. At least four clones of each construct were sequenced fully to confirm the correct sequence.

The Y1, Y2 and Y5 receptors from the rhesus monkey have been cloned previously (Gehlert et al., 2001). Using clones containing the coding sequence of the rhesus Y1 and Y2 receptors and the same genomic DNA as above as template for the
rhY5 receptor and the primer pairs: rhY1.fH (5’-AAGCTTAAGCTTACCATGAATTCAACATTATTTTCCAG-3’) and rhY1.NSrKS (5’-CCGCGGTACCAGTTTCTTTACATCATCATTGTTG-3’); rhY2.fH (5’-AAGCTTAAGCTTACCATGGGTCCAATAGGTACAGAGG-3’) and rhY2.NSrKS (5’-CCGCGGTACCAGTTTCTTGTTGAAG-3’); rhY5.fH (5’-AAGCTTAAGCTTACCATGGATTTAGAGCTCGATGAAT-3’) and rhY5.NSrKS (5’-CCGCGGTACCAGTTTCTTGTTGAAG-3’), the coding sequence of these receptors were amplified by PCR, cut and ligated into plasmid pRLUC-N2 as described for rhY4, generating the constructs rhY1-RLUC, rhY2-RLUC, and rhY5-RLUC. A fragment containing human opioid receptor δ was amplified from a plasmid using the primers hDEL.fH (5’-AAGCTTAAGCTTACCATGGAAACCGGCCCATCC-3’) and hDEL.NSrKS (5’-CCGCGGTACCAGGCGACGCGACGCCACC-3’), cut and ligated into pRLUC-N2 to generate hDELTA-RLUC. Similarly, the human opioid μ receptor was amplified using the primers hMU.fH (5’-AAGCTTAAGCTTACCATGGACAGCCGCTGCC-3’) and hMU.NSrKS (5’-CCGCGGTACCAGGCGACGCGACGCCACC-3’) to generate hMU-RLUC.

Expression of NPY family receptors in human embryonic kidney (HEK293) cells.

HEK293 cells were grown in 90 mm dishes in a DMEM:F-12 (3:1) mix (Gibco) supplemented with 5% FBS, 20 mM HEPES and Penicillin Streptomycin (Gibco) at 37°C in 5% CO₂. 2 μg DNA from the constructs rhY4-GFP and rhY4 (WT) as well as the uncut pGFP²-N2 and the control vector BRET+ (PekinElmer Life Sciences) were individually transfected into HEK293 cells using 5 μl FuGENE6 (Roche) diluted in 145
μl OptiMEM (Gibco) according to the manufacturer’s instructions. Seventy-two hours later the media was replaced with media containing Zeocin (100 µg/ml, Neosystems) for the GFP\textsuperscript{2} constructs and G418 (500 µg/ml, Gibco) for the RLUC and the WT (in pcDNA3.1(+)) constructs. After 2-3 weeks, individual clones were picked using a sterile pipette tip. Clones expressing rhY4-GFP\textsuperscript{2} or cytosolic GFP\textsuperscript{2} were selected based on fluorescence intensity using an inverted fluorescent microscope and, subsequently, sorted into six-well plates while rhY4 (WT) expressing clones were randomly picked from the plate. Subsequently, clones were tested for receptor expression using \textsuperscript{125}I-hPP (Perkin Elmer Life Science) as radioligand as described below.

**Receptor binding studies**

HEK293 cells stably expressing the rhesus Y4 (WT) receptor and a cell line expressing the recombinant rhesus Y4-GFP\textsuperscript{2} 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 \textsuperscript{125}I-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 Company, Sunnyvale, CA). Various concentrations of peptides and peptide analogs (American Peptide Company) or 1229U91 (Eli Lilly and Company, Indianapolis, IN) were added to the incubations to determine binding affinity. For saturation binding analysis, HEK293 cell homogenates containing the wild type and GFP\textsuperscript{2}-tagged Y4 receptors, were incubated with 12 different concentrations of \textsuperscript{125}I-hPP for 2 h at room temperature. The results were analyzed using the Prism software package.
Protein concentrations were measured using Coomassie Plus® Protein Assay Reagent (Pierce) using BSA standards.

**Agonist induced GTPγS binding**

Binding of $^{35}$S-GTPγS (Perkin Elmer Life Science) was determined as described previously (Xu et al., 2001). Briefly, 96-well Costar plates received 50 µl buffer, 50 µl drug, 50 µl $^{35}$S-GTPγS (final concentration 100 pM), and 50 µl of cell membranes (50 µg of protein). The final concentrations of reagents in the $^{35}$S-GTPγS binding assays were: 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10 mM MgCl$_2$ and 0.1% BSA. Incubations were conducted for 3 h at 25°C (steady state). A scintillation proximity assay using wheat germ agglutinin (WGA) beads was used to detect $^{35}$S-GTPγS binding. Basal activity was defined as binding in the absence of agonist.

**BRET$^2$ studies of co-expressed GFP- and RLUC-tagged receptors.**

HEK293 cells of the stable cell line rhY4-GFP$^2$ were plated out in six-well dishes (30 mm diameter, Falcon). At about 40 - 50% confluency, 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 pRLUC-N2 using 3 µl FuGENE6 diluted in 97 µl OptiMEM (Gibco) according to manufacturers instructions. After 72 h., the cells from each well were detached by washing with 1 ml ice-cold PBS using a pipette tip, transferred to a 1.5 ml eppendorff tube, spun in a micro-centrifuge at 5000 rpm for 2 min, and resuspended in 150 µl PBS (about 10000 cells/µl). Cell suspension (25 µl) was dispensed into each well of a 96-well plate (OptiPlate96, white, Packard Biosciences).
Immediately before counting the plate in a Fusion™ instrument (PekinElmer Life Sciences), 25 µl of modified coelenterazine (DeepBlueCTM, PekinElmer 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 (GFP^2 optimum). The BRET^2 ratio for each sample was calculated as follows: (Sample_515nm – BG_515nm)/(Sample_410nm – BG_410nm) - baseline. The baseline signal was defined as the BRET^2 ratio from the stable rhY4-GFP^2 cell line co-transfected 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, cell extract from a stable cell line expressing a cytosolic fusion protein (BRET+, PekinElmer Life Sciences) consisting of luciferase in the amino-terminus and GFP^2 in the carboxy-terminus, was run in every 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-GFP^2 in each assay were determined by adding 25 µl cell suspension to a black 96-well polypropylene plate (Nunc) and the fluorescence assessed using the Fusion™ instrument (excitation λ = 425 ± 20 nm, detection λ = 515 ± 30 nm).

*Effect of agonist stimulation on cells co-expressing Y4-GFP^2 and Y4-RLUC.*

Cells in 6-well plates were transfected as described above. These experiments were carried out 48-72 hours post-transfection. One hour prior to the experiment, one ml of media containing hPP, 1229U91, hNPy, hPYY, or h[D-Trp^32]NPY was added to the cells. The Y4 agonists hPP and 1229U91 were tested at final concentrations ranging
from 1 pM to 1 µM while hNPY, hPYY, or h[D-Trp^{32}]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 assessed using the Fusion instrument as described above. A time-course study was also performed by adding 100 nM hPP at various times before the BRET^{2} ratio was assayed. The effects of concanavalin A type III (0.5 mg/ml, Sigma), an inhibitor of clathrin-mediated internalization, and cycloheximide (100 µM, Sigma), a protein synthesis inhibitor, were also investigated by addition to the media 30 min prior to BRET^{2} assay.

Chemical cross-linking and Western blot analysis.
GFP^{2}-tagged Y4 receptors stably expressed in HEK293 cells were grown to confluence in 6-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 2mg/ml bis[sulfosuccinimidyl]suberate, BS^{3} (Pierce) 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^{®} LDS sample loading buffer (141 mM Tris Base, 106 mM Tris HCl, 2% LDS, 0.51 mM EDTA, 0.22 mM SERVA^{®} Blue G250, 0.175 mM Phenol Red, 10% Glycerol, pH 8.5) and heated for 5 min at 95°C. Electrophoresis was conducted using a Xcell II mini cell (Invitrogen) with 7% NuPAGE^{®} Tris-Acetate SDS-PAGE gels. Proteins were transferred onto PVDF membranes using an Xcell II Blot Module (Invitrogen). The presence of GFP^{2} tagged rhY4 receptors was detected using a 1:4000 dilution of a mouse monoclonal GFP antibody (Roche) and a
mouse WesternBreeze® Chemiluminescent Kit (Invitrogen). The gel was opposed to a Hyperfilm (High performance chemiluminescence film, Amersham Pharmacia Biotech) for 30 seconds. The film was developed and images were evaluated by densitometry using the MCID Elite 6.0 software (Imaging Research Inc.).
RESULTS

Sequence analyses of the rhY4 receptor.

The rhY4 receptor was cloned using PCR with primers based on the flanking sequences of the human Y4 (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 45-47 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, G716) and six (T70, C96, C348, T716). One of the polymorphisms resulted in a replacement of a polar amino acid to a highly hydrophobic amino acid; arginine239 (the same amino acid as in human Y4) -> leucine. This position is located at the beginning of the third intra-cellular loop, right after TM5. 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, G716).

Expression and pharmacological characterization.

The rhY4-GFP\(^2\) and rhY4 (WT) receptors expressed stably in HEK293 cells bound \(^{125}\)I-hPP according to a saturable one-site model with dissociation constants (\(K_d\)) of 65 ± 2.6 and 31 ± 1.7 pM, respectively and B-max values of 17709 ± 326 and 1593 ± 31 fmol/mg protein, respectively. A very high rhY4-GFP\(^2\) expressing cell line was selected to
maximize the fluorescent signal for subsequent BRET$^2$ studies. Eight different peptides were tested for inhibition of $^{125}$I-hPP binding at the rhY4-GFP$^2$ 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 ($r^2$) between the GFP$^2$-tagged receptor to the wildtype 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 difference in $K_d$-values seen for the radioligand. Functional coupling to G-proteins for the rhY4 (WT) receptor stably expressed in HEK293 cells was confirmed by $^{35}$S-GTP$\gamma$S binding. Human PP increased binding in a concentration-dependent manner with a pEC$_{50}$-value of 8.01 ± 0.16 nM (n=3, Fig. 2). HEK293 cells expressing the rhY4-GFP$^2$ chimeric protein were subjected to the same conditions but no agonist effect on $^{35}$S-GTP$\gamma$S binding could be monitored in these cells.

**BRET$^2$ studies of co-expressed GFP$^2$- and RLUC-tagged receptors.**

The BRET$^2$ ratios were assayed for the cloned rhY4 receptor co-expressed with the rhesus Y1, Y2, and Y5 receptors as well as the human $\delta$- and $\mu$-opioid receptors. The expression of the GFP$^2$-tagged rhY4 receptor (as determined by GFP$^2$-fluorescence) remained the same no matter which RLUC tagged construct was co-expressed with it. The expression levels of each of the RLUC tagged receptors were assayed by chemiluminescence. The Y2, Y4, $\delta$ and $\mu$ 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 BRET$^2$ ratios as the less related opioid δ and μ receptors of 0.035-0.065 whereas co-expression of rhY4-GFP$^2$ and rhY4-RLUC gave a value of 0.169 ± 0.009 (Fig. 3). As a positive control, BRET+, a cytosolic fusion protein with RLUC in the N-terminus and GFP$^2$ in the C-terminus, was used. Homogenate from a cell line stably expressing BRET+ gave a ratio of 0.50 ± 0.01 (n=9).

**Effect of agonist stimulation on cells expressing rhY4-GFP$^2$ and rhY4-RLUC.**

To assess the effects of agonist stimulation, cells expressing rhY4-GFP$^2$ 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 BRET$^2$ ratio. A time-course study revealed that the maximal decrease was reached within 15 minutes after addition of hPP at a final concentration of 100 nM (Fig. 4a). Both hPP and 1229U91 lowered the BRET$^2$ ratio in a concentration-dependent fashion (Fig. 5) with EC$_{50}$-values of 2.9 and 14 nM, respectively. In these experiments, the top BRET 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 of hNPY, hPYY, or the Y5-selective agonist h[D-Trp$^{32}$]NPY 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 BRET$^2$ ratio (Fig 4b). With both inhibitors the reduction in BRET$^2$ ratio was similar to that observed with the non-treated cells (45-49 % reduction). A small increase in both the unstimulated and agonist stimulated BRET$^2$ ratios were
observed with cycloheximide pre-treatment and a decrease with concanavalin A pretreatment compared to untreated cells (columns 1 and 2), possibly due to cell toxicity.

**Western blot analysis of rhY4-GFP\textsuperscript{2} receptors**

Rhesus Y4 receptors tagged with GFP\textsuperscript{2} were chemically cross-linked using the membrane impervious cross-linker, bis[succinimidyl]suberate (BS\textsuperscript{3}). Subsequent analysis of the unstimulated BS\textsuperscript{3} treated cells by non-reducing SDS-PAGE and Western blotting using a GFP specific antibody revealed two large molecular weight protein complexes, 80 and 160 kDa with relative optical densities (ROD) 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 \(\mu\)M hPP produced almost a 2-fold decrease in the 160 kDa band (ROD 0.54) (Fig. 6, Lane 4).
DISCUSSION

Our group has recently cloned the Y1, Y2, and Y5 (Gehlert et al., 2001) and the Y4 (Berglund et al., 2003b) 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 two, four, and two 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 (Arg^{239}/Leu^{239}), located in the third intracellular loop, while 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 no: XM_011916).

The pharmacology of the rhY4 receptor (Arg^{239}) was found to be indistinguishable from that of the hY4 (Bard et al., 1995; Lundell et al., 1995) suggesting similar ligand recognition domains. The wildtype rhY4 receptor was also found to couple to G-protein (Fig. 2) demonstrating that the receptor is functional when stably expressed in HEK293 cells. On the other hand, the GFP^{2}-tagged rhY4 receptor bound ^125^{I}-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 GFP^{2} at the C-terminus of the receptor may affect agonist affinity, but the
pharmacological properties remain intact. However, the lack of agonist effect on $^{35}\text{S-}\text{GTP}\gamma\text{S}$ at the rhY4-GFP$^2$ expressing cells suggests that the G-protein coupling is impaired in these cells. One possibility is that the presence of the 239 amino acid GFP$^2$ molecule attached to the C-terminus of the receptor affects coupling to G-proteins and thus shifts the equilibrium of the receptor-pool towards 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-GFP$^2$ 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., 1993). 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 BRET$^2$ technology. The main advantage of BRET$^2$ over its predecessor is the larger gap between the optimal wavelength for the luminescence from DeepBlueC™ and fluorescence from GFP$^2$ (105 nm) compared to earlier versions of BRET. High BRET$^2$ ratios, suggesting the close proximity (i. e. receptor dimerization) of GFP$^2$ 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 BS$^3$. 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-GFP\(^2\) monomer, while the 160 kDa protein is a homodimer. Similar to what has been shown for the δ opioid homodimer (Cvejic 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 non-reducing gel conditions suggests that covalent disulphide bonds are not involved in Y4 receptor dimerization contrary to what is known for the metabotropic glutamate receptor 5 and the muscarinic acetylcoline 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, very recently, using fluorescence resonance energy transfer (FRET) and fluorescence microscopy it was shown that the Y1, Y2, and Y5 receptors, when expressed individually in baby hamster kidney cells (BHK), form homodimers (Dinger et al., 2003). In the present study, the corrected BRET\(^2\) ratio for the Y4 homodimer (0.17) was almost three times higher than that found when Y4-GFP\(^2\) was co-expressed with Y2-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-GFP\(^2\) co-expressed 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 BRET
studies displayed a very high expression level of the rhY4-GFP. 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 while the rhY1 and rhY5 receptors displayed two- to three-fold lower expression. Yet, the BRET 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 BRET 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-family receptors, Y2 shows the lowest sequence identity to Y4 whereas the Y1 receptor that is highly similar to the Y4 receptor and displayed the lowest BRET ratio for heterodimerization with the Y4 receptor.

In contrast to the agonist-induced homodimers observed with the β2-adrenergic (Angers et al., 2000), the bradykinin B2 (AbdAlla et al., 1999), the thyrotropin-releasing hormone (Kroeger et al., 2001; Zhu et al., 2002), and the gonadotropin-releasing hormone (Kroeger et al., 2001) receptors, the rhY4 receptor complex dissociated in a concentration-dependent fashion when stimulated by the endogenous agonist, PP, or by the bridged anti-parallel 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[D-Trp32]NPY did not affect dimer formation. Therefore, agonist-induced dissociation of the dimer is a receptor-mediated response and not a nonspecific 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 CCK-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 one-hour stimulation by PP (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 BRET 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


Berglund MM, Schober DA, Statnick MA, McDonald PH and Gehlert DR (2003b) The Use of Bioluminescence Resonance Energy Transfer (BRET2) to Study Neuropeptide Y Receptor Agonist Induced {beta}-Arrestin 2 Interaction. *J Pharmacol Exp Ther* **306**:147-156.


Gehlert DR, Gackenheimer SL and Schober DA (1992) [Leu31-Pro34] neuropeptide Y identifies a subtype of 125I-labeled peptide YY binding sites in the rat brain. 


FOOTNOTES

This work was supported by Eli Lilly and Company.

Parts of this work have previously been presented as an abstract at the annual Neuroscience Meeting in Orlando 2002 (Abstract # 544:11).

Send reprint requests to: Dr. Donald R. Gehlert, Lilly Research Laboratories, LCC, Indianapolis, IN 46285. E-mail: gehlert_donald_r@lilly.com

1) Present address: Department of Assay Development and Screening, Biovitrum AB, SE-11276 Stockholm, Sweden
LEGENDS FOR FIGURES

Fig. 1
Correlation between rhY4-GFP² receptors expressed in HEK293 cells and the rhY4 (WT) receptors expressed in HEK293 cells. The correlation (r²) between pKi for rhY4-GFP² and the pKi for rhY4 (WT) was 0.97.

Fig. 2
Agonist induced G-protein coupling of the rhY4 receptor stably expressed in HEK293 cells. Various concentrations of hPP were incubated with 100 pM ³⁵S-GTPγS and 50µg tissue for 60 minutes. Following this incubation, 1 mg wheat germ agglutinin coated scintillation beads were added and the plate counted on a Wallac MicroBeta. The data are presented as Mean ± S. E. M. from three experiments performed in duplicate.

Fig. 3
BRET² ratios for the rhY4 receptor homodimer compared to heterodimers between rhY4 and rhesus Y1, Y2, Y5 and human opioid receptors δ and µ. 1 µg of each of the constructs expressing the RLUC-tagged receptors indicated were transiently transfected into HEK293 cells that stably expressed the rhY4-GFP² receptor. Ratios are expressed as Mean ± S. E. M. from five to nine experiments performed in quadruplicate.

Fig. 4
Dissociation of rhY4 homodimers by agonist stimulation. A) The effect of 100 nM hPP added to growth media 15, 30, 60, and 180 min. prior to BRET² assay. Maximal effect
was observed within 15 minutes of stimulation. Data are presented as Mean ± S. E. M. (n=3). B) Effect of inhibitors on agonist induced dissociation of Y4 homodimers. Concanavalin A, an inhibitor of clathrin mediated internalization, (0.5 mg/ml, columns 3 and 4) and Cycloheximide, a protein synthesis inhibitor, (100 µM, columns 5 and 6) were added to the growth media 30 min. before agonist (hPP at 100 nM). BRET² ratio was assayed 60 minutes after agonist stimulation. Data are presented as Mean ± S. E. M. (n=3).

Fig. 5
Concentration-response effect of agonist induced dissociation of the rhY4 receptor homodimer. Normalized BRET² ratios after 60 min stimulation by the Y4 agonists hPP (n=9) and 1229U91 (n=6) demonstrate a concentration-dependent dissociation of the rhY4 homodimer. In contrast, 100 nM hPYY, hNPY, and h[D-Trp³²]NPY did not reduce the BRET² signal. Data are presented as Mean ± S. E. M.

Fig. 6
Western blot analyses of HEK293 cells stably expressing rhY4-GFP². The cells were detached, cross-linked with the membrane impermeable agent BS³ (lanes 1, 3, and 4 and the controls). Two bands, one at MW=160 kDa, (dimerized and glycosylated rhY4-GFP²) and one of 80 kDa (monomer), are visual after BS³ incubation. Without BS³ the 80 kDa band is the only visible band. Three controls were run on the same gel: HEK293 cells stably expressing the rhY4 receptor without GFP (negative control) (lane 5), GFP² without receptor (lane 6), and the positive control RLUC-GFP² construct (BRET+) (lane
7). Western blot analysis was performed and the rhY4-GFP$^2$ receptors detected using a GFP antisera.
TABLES

Table 1

*Peptide analogs competing against \(^{125}\text{I}-\text{hPP}\) binding to HEK293 cells stably expressing the wild type and \text{GFP}^2\ tagged rhY4 receptor.*

Eleven point displacement curves were used to determine the affinity of various peptides or peptide analogs when competing with \(^{125}\text{I}-\text{hPP}\) binding to the wild type or \text{GFP}^2\ tagged rhY4 receptor. The data were best fit to a one-site model and \(K_i\) 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.).

<table>
<thead>
<tr>
<th>Peptide and Peptide Analogs</th>
<th>rhY4 (WT) Receptor</th>
<th>rhY4-GFP(^2) 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-Trp(^{32})]-NPY</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human [Leu(^{31}),Pro(^{34})]-NPY</td>
<td>0.261 ± 0.043</td>
<td>1.93 ± 0.26</td>
</tr>
<tr>
<td>Human NPY(3-36)</td>
<td>15.10 ± 1.78</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Human NPY(13-36)</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>
<tr>
<td>1229U91</td>
<td>0.0344 ± 0.007</td>
<td>0.233 ± 0.104</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2

Concentration hPP (logM) vs. (35S)-GTPγS Binding (%Control)
Fig. 3

Corrected BRET2 ratio

Receptor pair

rhY4-rhY1 (n=8)
rhY4-rhY2 (n=6)
rhY4-rhY4 (n=9)
rhY4-rhY5 (n=8)
rhY4-Delta (n=8)
rhY4-Mu (n=5)
Fig. 4

A

Corrected BRET$^2$ ratio

Unstimulated

100 nM hPP 15 min

100 nM hPP 30 min

100 nM hPP 60 min

100 nM hPP 180 min

B

Corrected BRET$^2$ ratio

Unstimulated

100 nM hPP

46%

Unstimulated

100 nM hPP

49%

Unstimulated

100 nM hPP

45%
Fig. 5
Fig. 6

Unstimulated crosslinked

Unstimulated

hPP (10 nM)

hPP (1 µM)

rhY4 without GFP

GFP (non-fused)

BRET +