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Fluorescence- and luminescence-based methods for the determination of affinity and activity of neuropeptide Y_2 receptor ligands ☆

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Abstract

With respect to the discovery and characterization of neuropeptide Y_2 receptor ligands as pharmacological tools or potential drugs, fluorescence- and luminescence-based assays were developed to determine both the affinity and the activity of receptor agonists and antagonists. A flow cytometric binding assay is described for the hY_2 receptor stably expressed in CHO cells using cy5-labeled porcine neuropeptide Y and compared with a radioligand binding assay. Binding of the fluorescent ligand was visualized by confocal microscopy. Stable co-transfection with the chimeric G protein Gq_{i5} enabled the establishment of a spectrofluorimetric fura-2 and a flow cytometric fluo-4 calcium assay. Further stable expression of apoaequorin targeted to the mitochondria allowed the establishment of an aequorin assay which could be performed in the 96-well format. The shape of the concentration–response curves of porcine neuropeptide Y in the presence of the Y_2-selective receptor antagonist BIIE0246, characteristic of either competitive or insurmountable antagonism, depended on the period of incubation with the cells. Functional data of Y_2 receptor agonists and antagonists determined in the fluorescence- and luminescence-based assays were in good agreement.

Keywords: Fluorescent probe; Neuropeptide Y; Y_2 receptor; Flow cytometry; Chimeric G protein; Aequorin; BIIE0246

1. Introduction

Neuropeptide Y is a member of the so-called pancreatic polypeptide or neuropeptide Y family that also includes peptide YY and pancreatic polypeptide (Michel et al., 1998). Neuropeptide Y is widely distributed in the brain and the peripheral nervous system, and is implicated in various physiological processes including regulation of food intake, anxiety, mood and memory, blood pressure and circadian rhythm. Neuropeptide Y and related peptides exert their biological actions by interacting with at least five different G protein-coupled receptors, designated Y_1, Y_2, Y_4, Y_5 and y_6 (Hazelwood, 1993; Michel, 2004). Their main signal transduction pathway is a coupling to pertussis toxin sensitive G proteins of the $G_{i/o}$ family, leading to an inhibition of adenylyl cyclase.

The neuropeptide Y_2 receptor is considered the most abundant neuropeptide Y receptor in the human brain and to be involved, for instance, in memory and learning. As recent studies reported on an anorectic effect of the Y_2 preferring agonist peptide YY(3–36) after peripheral application in rodents and humans (Abbott et al., 2005; Batterham et al., 2002), the Y_2 receptor has also become an attractive drug target for the treatment of eating disorders.

Binding data of Y_2 receptor ligands are usually determined in radioligand binding assays, requiring a filtration step in order to separate bound from unbound ligand. Although homogenous binding assays using the scintillation proximity assay technique have been described (Dautzenberg, 2005; Dautzenberg et al., 2005), the use of radio-labeled ligands is still indispensable,

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causing high costs and radioactive waste. Recently, BODIPY-labeled neuropeptide Y analogues with high affinity and selectivity for the Y_1, Y_2, Y_4 and Y_5 receptors have been described (Dumont et al., 2005). The use of fluorescent ligands in a flow cytometric binding assay has been previously described for the chemokine receptor CXCR4 (Hatse et al., 2004), the epidermal growth factor (Stein et al., 2001) and the formylpeptide receptor (Edwards et al., 2005).

As a functional assay, the establishment of calcium mobilization by co-transfection of neuropeptide Y receptors and chimeric G proteins Gq_{o5}, Gq_{i5} and Gq_{i9} into HEK293 cells has been reported (fluorometric imaging plate reader (FLIPR) assay) (Dautzenberg et al., 2005). However, calcium mobilization assays using non-ratiometric fluorescent indicator dyes like fluo-4 have the drawback of dye leakage and the use of ratiometric indicator dyes such as fura-2 is often not amenable to the application in the multiplate reader format.

The photoprotein aequorin has been widely used for many years to visualize changes in intracellular calcium (Blinks, 1978), but the purified protein had to be microinjected, limiting its use as a calcium indicator. The cloning of the apoaequorin cDNA (Inouye et al., 1985) and the recombinant expression of the protein by various cell types has greatly improved the use of the bioluminescent protein. Reconstitution of aequorin can be accomplished by simple addition of the co-factor coelenterazine to the cell culture medium (Torfs et al., 2002). In contrast to fluorescence indicator dyes used at high concentrations (usually 20–200 μM) aequorin (usually recombinantly expressed <1 μM) does not significantly affect endogenous Ca^{2+} buffer capacity (Brini et al., 1995), and no ester hydrolysis products, which may alter the physiological response, are released in the cell. Therefore, recombinantly expressed aequorin has been often used for the functional screening of various G protein-coupled receptors (Button and Brownstein, 1993; Dupriez et al., 2002; Le Poul et al., 2002; Schaeffer et al., 1999; Stables et al., 1997; Torfs et al., 2002; Ungrin et al., 1999). The most robust bioluminescence signals after receptor activation were obtained with mitochondrially targeted aequorin (Stables et al., 1997, 2000). The use of cells stably co-expressing mitochondrial apoaequorin, the promiscuous Galpha16 protein and various G protein-coupled receptors have been previously described (Dupriez et al., 2002; Stables et al., 1997).

Here we report on the establishment of a flow cytometric binding assay and the stepwise stable transfection of cells with the *Gq_{i5}* and *mtAEQ* constructs for the development of functional fluorescence- and luminescence-based assays for the neuropeptide Y_2 receptor.

2. Materials and methods

2.1. Materials, peptides, reagents and radiochemicals

The peptides porcine neuropeptide Y, porcine [L^{31}, P^{34}]-neuropeptide Y and porcine neuropeptide Y(13–36) were synthesized as described previously (Cabrele et al., 2001). The peptides were used with a purity higher than 90% as determined by analytical HPLC. Porcine peptide YY was purchased from Novabiochem, Switzerland. Porcine [^{3}H]propionyl-neuropeptide Y (specific activity 2.07, 3.96 TBq/mmol respectively) was from Amersham Biosciences (Little Chalfont, UK). The vectors pcDNA3.1/hygro (Invitrogen) and pcDNA3.1/zeo (Invitrogen) were kindly provided by Dr. T. Dobner, University of Regensburg, Germany. The pcDNA3-hY_2 expression vector was a gift from Dr. P. Rose, Bristol-Myers Squibb, Princeton, New Jersey, USA. The cDNA encoding the chimeric G protein Gq_{i5} in pcDNA1 was kindly provided by Prof. Dr. Bruce Conklin, Gladstone Institute of Cardiovascular Disease, University of California, San Francisco, USA. After introduction of an *Eco*RV site at the 3′-end by PCR, the construct was subcloned into the *Bam*HI/*Eco*RV cassette of the pcDNA3.1/hygro vector. The pMTAEQ (Molecular Probes) vector was a gift from Prof. Dr. S. Thayer, University of Minnesota, USA. The construct encoding apoaequorin targeted to the mitochondria was subcloned into the *Eco*RI site of pcDNA3.1/zeo.

Fura-2-AM and Fluo-4-AM were purchased from Molecular Probes, Eugene, Oregon, USA. The cyanine dye cy5 was purchased from Amersham Biosciences (Little Chalfont, UK). Coelenterazine h was purchased from Biotium, Hayward, CA, USA. BIIE0246 (**1**) (Doods et al., 1999) and compounds **2** (Dollinger et al., 1999) and **3** (Fig. 1) were synthesized in our laboratory. Structures were confirmed by NMR and mass spectrometry and the purity was checked by analytical HPLC and elemental analysis (results for C, H, N were within ±0.4% from theoretical values).

2.2. Cell culture and stable transfections

CHO-K1 cells were grown in Nutrient Mixture Ham's F12 medium supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany). All transfections were carried out using the FuGENE6 reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. To generate the stable cell line CHO-hY_2, the cells were transfected with the pcDNA3-hY_2 vector. 2 days after transfection, selection was initiated by the addition of G418 (400 μg/ml) to the medium. After 2 weeks of selection, single separately growing cell

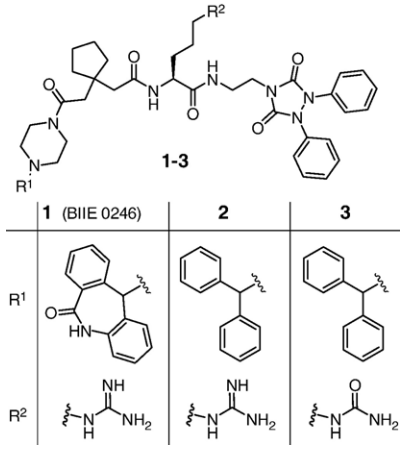

Fig. 1. Structural formulas of Y_2 receptor antagonists.

colonies were isolated, expanded and tested for specific binding of cy5-labeled porcine neuropeptide Y by flow cytometry (see below).

CHO-hY_2 cells were transfected with the pcDNA3.1/hygro-Gq_{i5} vector, and cells were selected by the addition of hygromycin (400 μg/ml) to the medium 2 days after transfection. Stable CHO-hY_2-Gq_{i5} cell clones were selected and tested for robust response in the flow cytometric calcium assay.

CHO-hY_2-Gq_{i5} cells were transfected with the pcDNA3.1/zeo-mtAEQ vector and selected with 250 μg/ml zeocin 2 days after transfection. Single cell clones were selected on the basis of their functional response to porcine neuropeptide Y in the aequorin assay.

2.3. Synthesis of cy5-labeled porcine neuropeptide Y

For the labeling reaction, 0.5 mg of porcine neuropeptide Y were dissolved in 20 μl of dimethyl sulfoxide and 250 μl of 0.1 M sodium bicarbonate buffer (pH 9.5) containing 33% acetonitrile were added. One portion of cy5 succinimidyl ester (ca. 0.2 mg) was dissolved in 20 μl of anhydrous dimethyl sulfoxide and added to the peptide solution. After 3 h of incubation at room temperature the labeled porcine neuropeptide Y was purified by HPLC using a Nucleosil 300-5 C-18 column (Macherey-Nagel, Düren, Germany). The gradient was formed by a mixture of solvent A containing 60% of acetonitrile and 40% of 0.1% aqueous trifluoroacetic acid (TFA), and solvent B consisting of 0.1% aqueous TFA. The fraction of solvent A was raised from 50% to 80% over 40 min in a linear manner at a flow rate of 1 ml/min. The labeled peptide indicated by the concomitant increase in absorption (649 nm) and tyrosine fluorescence (λ_{ex}=275 nm, λ_{em}=305 nm) was collected manually, and the concentration was determined spectrophotometrically using the molar absorption coefficient ε_{649}=310,000 M^{-1} cm^{-1} (in mobile phase at the retention time of the product, i. e. 18 min).

2.4. Flow cytometric binding assay

The cells were grown to 80–90% confluency, trypsinized and resuspended in Ham's F12 medium, containing 10% fetal calf serum for the inactivation of trypsin. Cells were counted, centrifuged for 5 min at 300 g and resuspended at a density of 10^6 cells/ml in binding buffer (25 mM HEPES, 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$, 1% bovine serum albumine (BSA), 0.1 mg/ml bacitracin, pH 7.4). All binding assays were performed in a final volume of 500 μl containing cy5-labeled porcine neuropeptide Y and unlabeled peptide or competitor as needed. Saturation assays were performed in the presence of increasing concentrations of cy5-labeled porcine neuropeptide Y while competition binding experiments were carried out using 5 nM of cy5-labeled porcine neuropeptide Y in the presence and absence of various competitors at concentrations ranging from 10^{-12} to 10^{-5} M. Unspecific binding was determined in the presence of 1 μM of porcine neuropeptide Y. The samples were incubated in siliconized reaction vessels for 90–120 min at room temperature and analyzed by a FacsCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany) without further processing. Settings were: FSC=E-1, SSC=280, FL-4=800, flow rate: high. The measurement stopped when 20,000 gated events were counted. The geometric means of fluorescence in channel FL-4 of the gated cells were calculated using the WinMDI 2.8 software.

The constant K_i for the inhibition of the binding of cy5-labeled porcine neuropeptide Y by unlabeled competitors was calculated from the concentration of unlabeled competitor, producing 50% inhibition (IC_{50}) of the specific binding of the labeled peptide using the following relation $K_i = IC_{50} \cdot [K_d/(K_d+L)]$ where K_d is the dissociation constant and L the concentration of cy5-labeled porcine neuropeptide Y (Cheng and Prusoff, 1973).

2.5. Radioligand binding assay

CHO-hY_2-Gq_{i5}-mtAEQ cells were seeded at a density of 25,000 cells/well in 500 μl Ham's F12 medium plus 10% fetal calf serum on 24-well plates 2 days before the experiment. The medium was removed by suction, and 200 μl of binding buffer were added. 25 μl of competitor or unlabeled peptide and 25 μl of the radioligand in binding buffer were added. Cells were incubated at room temperature under slight shaking for 2 h. Then, the supernatant was sucked off, and the adherent cells were washed twice with ice-cold buffer. Lysis of the cells was accomplished by the addition of 200 μl of lysis buffer, containing 8 M urea (Merck, Darmstadt, Germany), 3 M acetic acid (Merck) and 1% Triton-X-100 (Sigma, München, Germany), followed by 30 min incubation at room temperature under slight shaking. The lysates were transferred into scintillation vials and each well was washed with another 200 μl of lysis buffer. Radioactivity was measured in a LS 1801 β-counter (Beckmann Instruments, München, Germany). Unspecific binding was determined in the presence of 1 μM of unlabeled porcine neuropeptide Y. Assays were performed in triplicate.

2.6. Flow cytometric calcium assay

Cells were grown to 70–90% confluency, trypsinized and detached with Ham's F12 supplemented with 10% fetal calf serum. Cells were centrifuged at 300 ×g at room temperature for 5 min and resuspended at a density of $2 \cdot 10^6$ cells/ml in loading buffer (120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM HEPES and 10 mM glucose at pH 7.4) supplemented with 0.7 μM fluo-4-AM, 0.02% pluronic™ and 1.5% BSA. The cells were incubated in the dark for 30 min at room temperature and recentrifuged at 300 ×g for 5 min. After resuspension in loading buffer at a density of $0.5–1 \cdot 10^6$ cells/ml, the cells were again incubated for 30 min at room temperature in the dark. Measurements were performed in a purpose-build glass tube closed by a silicon septum as described (Schneider, 2005). This instrumentation allows injections into the samples during continuous flow cytometric measurements. After 30 s of measurement of the basal fluorescence under permanent stirring 10 μl of agonist solution were injected to 1 ml of the cell suspension with a Hamilton syringe and data were recorded for another 90 s. Receptor antagonist solutions were added to the cell suspension 1 min before the calcium signal was elicited with 70 nM porcine neuropeptide Y.

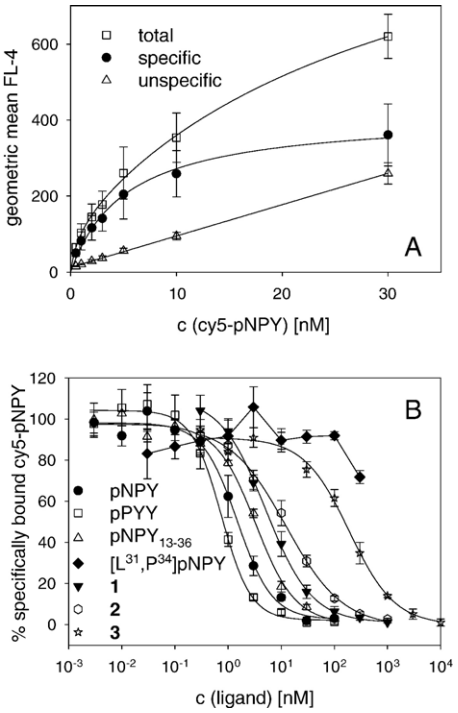

Fig. 2. Flow cytometric binding assay with CHO cells stably expressing the hY_2 receptor. Panel A: saturation experiment using increasing concentrations of cy5-labeled porcine neuropeptide Y. Panel B: competition assay with various ligands in the presence of 5 nM cy5-labeled porcine neuropeptide Y. Unspecific binding was determined in the presence of 1 μM porcine neuropeptide Y. Mean values ± S.E.M, n=3–5. (cy5-pNPY: cyanine5-labeled porcine neuropeptide Y; pNPY: porcine neuropeptide Y; pPYY: porcine peptide YY; $pNPY_{13–36}$: porcine neuropeptide Y (13–36); [L^{31}, P^{34}]pNPY: porcine [L^{31}, P^{34}]-neuropeptide Y).

Raw data were averaged with the WinMDI 2.8 and the SigmaPlot 8.0 software. The level of increase in fluorescence was calculated from the difference between the baseline (mean fluorescence of the first 25 s) and the highest value of the averaged curve. These amplitudes of the averaged signals were used to construct concentration–response curves. Every third measurement was a 100% reference signal elicited with 1 μM (for the determination of EC_{50} values) or 70 nM (inhibition curves) porcine neuropeptide Y. Instrument settings were: FSC: E-1; SSC: 280; FL-1: 350; flow: high.

2.7. Spectrofluorimetric calcium assay

The spectrofluorimetric fura-2 assay was performed as described elsewhere (Müller et al., 1997).

2.8. Aequorin assay

Cells were grown to 80–90% confluency, trypsinized and detached with Ham's F12 supplemented with 10% fetal calf serum. After centrifugation at 300 ×g for 5 min, the cells were resuspended in DMEM without phenol red supplemented with 1% fetal calf serum at a density of 10^7 cells/ml. Coelenterazine h (Biotrend, Köln, Germany) (1 mM stock solution in methanol) was added to a final concentration of 2 μM, and reconstitution of the holoenzyme was accomplished by incubation for 2 h at room temperature under gentle stirring in the dark. Cell suspensions were diluted with loading buffer to $5 \cdot 10^5$ cells per ml and incubated at room temperature for 3 h in the dark. 162 μl of the cell suspension were injected to 18 μl of peptide agonist in loading buffer supplemented with 1% BSA and 100 μg/ml bacitracin in a white 96-well plate (Greiner Bio-One, Frickenhausen, Germany) and the emitted luminescence (peak 1) was recorded in the kinetic mode for 43 s as a series of 200 ms integrations with a GENios Pro plate reader (Tecan, Salzburg, Austria). Subsequently, 20 μl of a 1% Triton-X-100 solution were injected to discharge the remaining aequorin, and light emission was recorded for additional 22 s (peak 2). For the characterization of antagonists, the cells were incubated in the presence of the antagonist for 1 h. 18 μl of 700 nM porcine neuropeptide Y were injected to 162 μl of the cell suspension, and luminescence (peak 1) was recorded for 43 s before the cells were lysed by the injection of 20 μl of Triton-X-100 (1% in binding buffer) (peak 2). The fractional luminescence was calculated by dividing the area of peak 1 by the total area under peaks 1 and 2 using the SigmaPlot 8.0 software.

2.9. Confocal microscopy

The cells were seeded in 200 μl Ham's F12 medium containing 10% fetal calf serum on a Lab-Tek™ II, 8 chamber

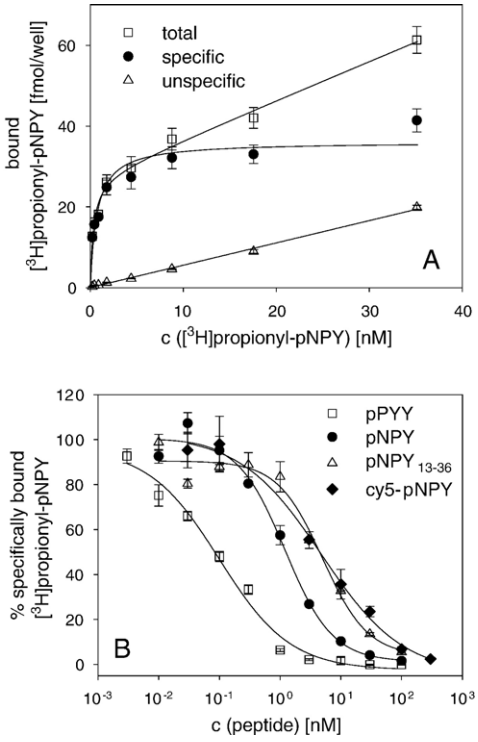

Fig. 3. Radioligand binding assay with CHO-hY_2-Gq_{i5}-mtAEQ cells. Panel A: saturation experiment using increasing concentrations of [^{3}H]-labeled porcine neuropeptide Y ([^{3}H]propionyl-pNPY). Panel B: competition assay with various ligands in the presence of [^{3}H]-labeled porcine neuropeptide Y. Unspecific binding was determined in the presence of 1 μM porcine neuropeptide Y. Mean values ± S.E.M, n=3. (cy5-pNPY: cyanine5-labeled porcine neuropeptide Y; pNPY: porcine neuropeptide Y; pPYY: porcine peptide YY; $pNPY_{13–36}$: porcine neuropeptide Y(13–36)).

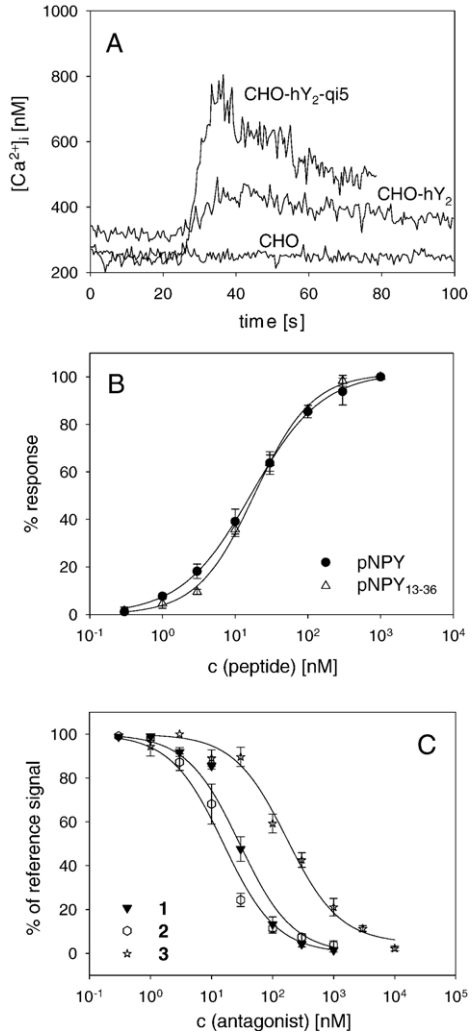

Fig. 4. Calcium responses in the fura-2 assay. Panel A: comparison of wild-type and transfected CHO cells with respect to the response to 50 nM porcine neuropeptide Y in the fura-2 assay. Panel B: concentration–response curves of Y_2 receptor agonists determined with CHO-hY_2-Gq_{i5}-mtAEQ cells. Panel C: inhibition of the neuropeptide Y-induced (70 nM) calcium signal by Y_2 receptor antagonists determined with CHO-hY_2-Gq_{i5} cells. Mean values ± S.E.M, $n = 3$. (pNPY: porcine neuropeptide Y; p$NPY_{13–36}$: porcine neuropeptide Y (13–36)).

coverglass system (Nalge Nunc, Naperville, IL, USA) 2 days prior to the experiment and were grown to 50–70% confluence. The medium was replaced with 200 µl of L-15 Leibowitz medium (Sigma) containing 10 nM cy5-labeled porcine neuropeptide Y and incubated at room temperature for 30 min. For the visualization of unspecific binding, 1 µM of unlabeled porcine neuropeptide Y was added to the incubation medium. Cells were washed with phosphate buffered saline (PBS) and fixed with paraformaldehyde (4% in PBS) for 30 min. Nuclei were stained with 500 nM Sytox® Green (Invitrogen, Karlruhe, Germany) in PBS for additional 30 min at room temperature, followed by a washing step with PBS. Confocal microscopy was performed with a Zeiss Axiovert 200 M microscope, equipped with the LSM 510 laser scanner, using a Plan-Apochromat 63×/1.4 objective with oil immersion. For the excitation of the cyanine dye, the 633 nm laser line was used, and fluorescence was detected using the 650 nm longpass filter. The nuclear dye Sytox® Green was excited at 488 nm and emitted fluorescence was detected using a 505 nm longpass filter. The scanning mode was multitrack.

2.10. Imaging with CCD camera

The cells were prepared as described for the aequorin assay. 150 µl of the cell suspension were added to 50 µl of porcine neuropeptide Y solution in a black 96-well plate (Greiner Bio-One, Frickenhausen, Germany) at once per row with a multichannel pipette and the plate was immediately transferred into a darkbox. Recording was started with a Hamamatsu 1394 ORCA-II BTA 512 cooled CCD camera; settings were: gain: 2, exposure: 60 s, binning: 4 (16 bit).

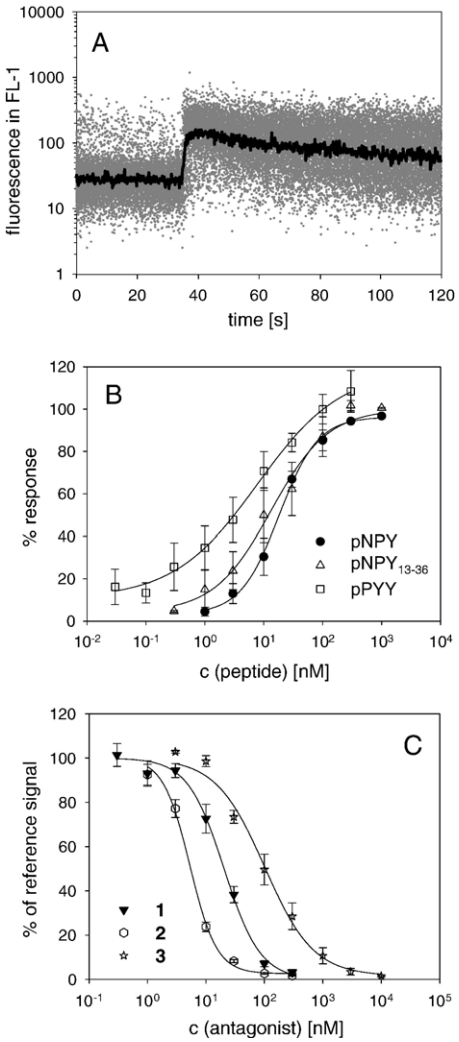

Fig. 5. Calcium responses in the flow cytometric fluo-4 assay using CHO-hY_2-Gq_{i5} cells. Panel A: calcium signal of 100 nM porcine neuropeptide Y. The grey dots represent the fluorescence of the gated cells. Panel B: concentration–response curves of Y_2 receptor agonists. Panel C: inhibition of the neuropeptide Y-induced (70 nM) calcium signal by Y_2 receptor antagonists. Mean values ± S.E.M, $n = 3$. (pNPY: porcine neuropeptide Y; pPYY: porcine peptide YY; p$NPY_{13–36}$: porcine neuropeptide Y (13–36)).

3. Results

3.1. Flow cytometric binding assay

CHO cells stably expressing the hY_2 receptor bound the fluorescent ligand cy5-labeled porcine neuropeptide Y with high affinity (K_d=5.2±2.1 nM; Fig. 2). Thus, 5 nM of the fluorescent ligand were used for competition binding experiments. The peptides porcine peptide YY (K_i=0.4±0.1 nM), porcine neuropeptide Y (K_i=0.8±0.2 nM), porcine neuropeptide Y(13–36) (K_i=1.7±0.4 nM) bound to the CHO-hY_2 cells with typical Y_2 receptor pharmacology, whereas porcine [L^{31}, P^{34}]-neuropeptide Y, a ligand with known high affinity for the Y_1 and moderate affinity for the Y_4 and Y_5 receptors, did not bind up to a concentration of 100 nM. The Y_2 receptor antagonist BIIE0246 (**1**) displaced the fluorescent ligand with high affinity (K_i=2.6±1.2 nM) whereas the structural analogues **2** (K_i=6.8±2.3 nM) and **3** (K_i=976.2±250.8 nM) bound with lower affinity.

3.2. Radioligand binding assay

The CHO-hY_2-Gq_{i5}-mtAEQ cells bound the radioligand [^{3}H]-labeled porcine neuropeptide Y with high affinity (K_d=0.7±0.2 nM; Fig. 3). The peptides porcine peptide YY (K_i=0.06±0.01 nM), porcine neuropeptide Y (K_i=0.4±0.1 nM) and porcine neuropeptide Y(13–36) (K_i=1.7±0.4 nM) showed the same pharmacological profile as determined in the flow cytometric binding assay. The affinity of cy5-labeled porcine neuropeptide Y (K_i=3.0±1.3 nM) was in the same range as determined by flow cytometry. The compounds **1**, **2** and **3** were not analyzed due to high adsorption to the assay materials.

3.3. Fluorescence-based calcium assays

CHO cells stably expressing only the hY_2 receptor responded poorly upon receptor activation. Stable co-transfection with the hY_2 and the Gq_{i5} gene enhanced the calcium response elicited by 50 nM porcine neuropeptide Y by 3-fold (Fig. 4A). Therefore, a spectrofluorimetric calcium assay became feasible using fura-2 loaded cells. Peptide agonists porcine neuropeptide Y and porcine neuropeptide Y(13–36) induced an increase in intracellular calcium concentration with potencies in the nanomolar range. The calcium signal induced by 70 nM porcine neuropeptide Y was inhibited by Y_2 receptor antagonists as shown in Fig. 4C.

Loading of the cells with fluo-4 allowed the detection of an increase in fluorescence after agonist stimulation by flow cytometry. As shown in Fig. 5A, the calcium response can be described by a fluorescence intensity vs. time dot plot. Relative increases in fluorescence were used to construct concentration–response curves of peptide agonists. The increase in fluorescence was suppressed by preincubation of the cells in the presence of Y_2 receptor antagonists and IC_{50} values were determined (Fig. 5, Table 1).

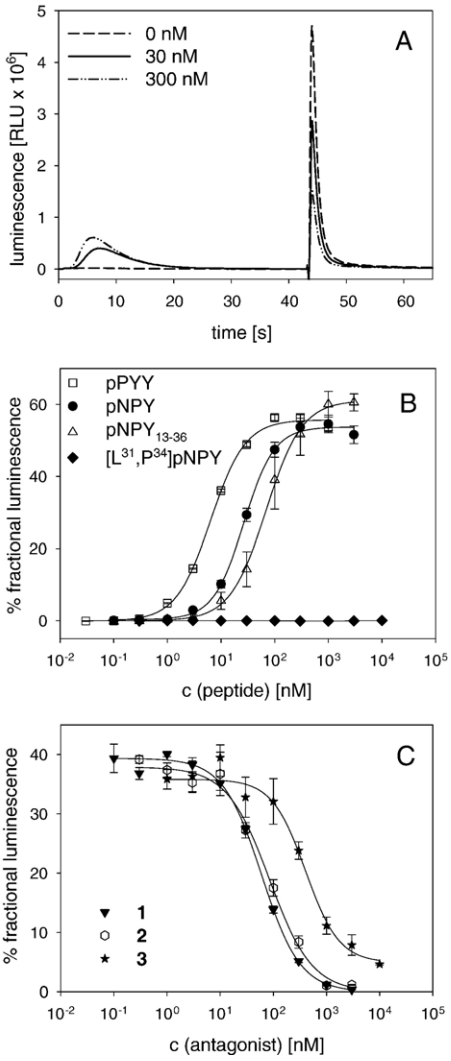

Fig. 6. Aequorin assay with CHO-hY_2-Gq_{i5}-mtAEQ cells. Panel A: luminescence signals after injection of the cells to porcine neuropeptide Y (first peak) and additional injection of 0.1% Triton-X-100 (second peak). Panel B: concentration–response curves of Y_2 receptor agonists. Panel C: inhibition of the neuropeptide Y-induced (70 nM) calcium signal by Y_2 receptor antagonists. Mean values±S.E.M, n=3. (pNPY: porcine neuropeptide Y; pPYY: porcine peptide YY; $pNPY_{13-36}$: porcine neuropeptide Y(13–36); [L^{31}, P^{34}]pNPY: porcine [L^{31}, P^{34}]-neuropeptide Y).

Table 1
Comparison of various receptor agonists and antagonists in the fluorescence-based fura-2 and fluo-4 assays, and in the aequorin assay

Y_2 receptor ligand	Flow cytometry (fluo-4)	Spectrofluorimetry (fura-2)	Aequorin assay
Porcine neuropeptide Y	18.1±2.9[a]	16.9±2.5[a]	30.9±2.2[a]
Porcine neuropeptide Y (13–36)	13.3±6.2[a]	18.6±1.5[a]	58.3±9.9[a]
Porcine peptide YY	7.9±5.5[a]	ND[b]	8.8±2.4[a]
1	20.4±2.9[c]	28.9±2.0[c]	50.9±12.9[c]
2	5.3±0.5[c]	14.5±1.5[c]	73.4±6.1[c]
3	101.1±18.0[c]	201.4±37.3[c]	359.4±23.8[c]

[a]EC_{50} [nM]; [b]ND: not determined; [c]IC_{50} [nM], calcium mobilization induced with 70 nM porcine neuropeptide Y. [a,c]The EC_{50} and IC_{50} values represent the means±S.E.M of three independent experiments.

3.4. Aequorin assay

Additional transfection leading to stable expression of apoaequorin allowed the conversion of the calcium signal into a luminescence signal. After reconstitution of the holoenzyme by addition of coelenterazine h, the cells responded with concentration-dependent increases of emitted light upon receptor activation (Fig. 6). EC_{50} values of peptide agonists (Fig. 6, Table 1) were in the same range compared to the results from the fura-2 and fluo-4 assay. As expected, porcine-[L^{31}, P^{34}]-neuropeptide Y did not elicit a response in the aequorin assay. The luminescence signal elicited with 70 nM porcine neuropeptide Y was inhibited by Y_2 receptor antagonists preincubated with the cells for 1 h prior to the injection of the receptor agonist. The determined IC_{50} values were slightly higher compared to the results from the fluorescence-based calcium assays (Table 1). Moreover, compound **1** appears to be about as potent as compound **2**, whereas compound **2** was superior in the fluorescence-based calcium assays. Presumably, this discrepancy results from the different experimental conditions. For instance, the incubation period was 60 min in the aequorin assay but only 1 min in the fluorescence-based calcium assays, and the lipophilic compounds may adsorb to different extent to the materials (glass vs. synthetic polymers) used in the different assays.

3.5. Confocal microscopy and luminescence detection by CCD camera

Binding of the fluorescent ligand cy5-labeled porcine neuropeptide Y was visualized by confocal microscopy. The ligand enriched at the cell membrane, whereas the binding was abolished in the presence of 1 μM unlabeled porcine neuropeptide Y. Concentration-dependent aequorin luminescence was detectable by a CCD camera as shown in Fig. 7.

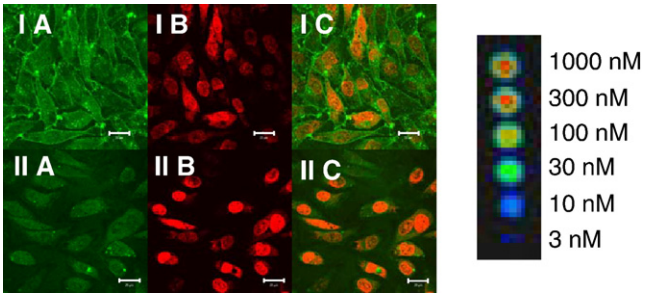

Fig. 7. Left panel: binding of cy5-labeled porcine neuropeptide Y to CHO-hY_2-Gq_{i5}-mtAEQ cells visualized by confocal laser scanning microscopy. In IA-C, the cells were incubated with 10 nM cy5-labeled porcine neuropeptide Y (A, green) for 30 min, washed with PBS, and fixed with paraformaldehyde (4% in PBS) for 30 min. Nuclei (B, red) were stained with 500 nM Sytox Green in PBS for additional 30 min, followed by a washing step with PBS. Merged images are shown in C. Unspecific binding (IIA-C) was measured after co-incubation of 10 nM cy5-labeled porcine neuropeptide Y with unlabeled porcine neuropeptide Y (1 μM). The white bars indicate 20 μm. Right panel: luminescence signals of CHO-hY_2-Gq_{i5}-mtAEQ cells in response to porcine neuropeptide Y, detected by a CCD camera. Warm colors indicate increasing light emission. The numbers represent the applied concentrations of porcine neuropeptide Y.

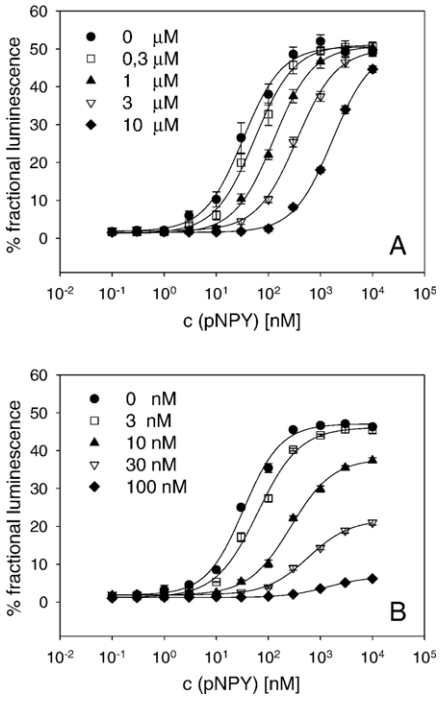

Fig. 8. Effects of BIIE0246 on the porcine neuropeptide Y-induced luminescence signals after co-application of porcine neuropeptide Y (panel A) or after preincubation of the cells in the presence of the receptor antagonist (**1**) for 1 h (panel B). Mean values±S.E.M, $n=3$. (pNPY: porcine neuropeptide Y).

3.6. Antagonism of BIIE0246 (*1*)

As recent studies with the Y_2 receptor antagonist BIIE0246 (Dautzenberg and Neysari, 2005) described an insurmountable antagonism (Neubig et al., 2003) after preincubation with the cells for 30 min, the effect of the preincubation was analyzed in the aequorin assay. In fact, when the cells were injected directly to the solution containing receptor agonist and antagonist, the concentration–response curves of porcine neuropeptide Y were shifted rightward, typical for competitive antagonism (Fig. 8). After preincubation of the cells in the presence of the receptor antagonist, the curves were also shifted to the right but with a concomitant depression of the maximum signal indicating an insurmountable fashion of the antagonism (Fig. 8).

4. Discussion

The use of flow cytometry has been shown to be a valuable tool for the determination of receptor binding data (Edwards et al., 2004). In this study, we established a flow cytometric binding assay for the hY_2 receptor performed in equilibrium without the necessity of separating bound from unbound ligand. As the natural ligand neuropeptide Y is a bulky peptide, the coupling to the fluorescent dye cy5 is well tolerated, and the labeled peptide retains a reasonable affinity for the hY_2 receptor stably expressed by CHO cells. The use of whole cells enabled us to determine binding constants of the receptor in its natural environment. Gating of the cell population prevents the measurement of fluorescent ligand bound to cell fragments or other

particles. The results were in good agreement with binding data determined in a conventional radioligand binding assay.

For the determination of functional data of Y_2 receptor ligands, the most commonly used assay is based on the inhibition of forskolin-induced cAMP formation (Beck-Sickinger et al., 1992; Goumain et al., 2001) as the Y_2 receptor is known to couple to the $G_{i/o}$ pathway (Michel et al., 1998). Reports on direct coupling of Y_2 receptor activation to an increase in intracellular calcium are controversial. Intracellular calcium mobilization in hY_2 transfected HEK293 cells was described by Gerald et al. (1995) but not observed by Dautzenberg et al. (2005). For CHP-234 cells (Lynch et al., 1994) and CHO cells transfected with the hY_2 gene (Rose et al., 1995) calcium responses were reported. By contrast, we measured only a slight increase in $[Ca^{2+}]_i$ in CHO cells stably expressing the hY_2 receptor in response to porcine neuropeptide Y. Therefore, we co-expressed the hY_2 receptor and the chimeric G protein Gq_{i5} (Coward et al., 1999) to obtain robust transient calcium mobilization, as this was already done successfully by Dautzenberg et al. (2005) in HEK 293 cells. The co-transfected CHO cells showed an improved calcium response upon receptor stimulation and a spectrofluorimetric fura-2 calcium assay was established. Peptide ligands showed the typical Y_2 receptor pharmacology with the rank order of potency porcine peptide YY > porcine neuropeptide Y > porcine neuropeptide Y(13–36) ≫ porcine [L^{31}, P^{34}]-neuropeptide Y. Loading of the cells with fluo-4 enabled the measurement of calcium mobilization by flow cytometry. By gating of the intact cells the signal to noise ratio is improved as not-gated cell fragments and particles with adsorbed fluorescent dye are not included to the calcium measurement. The determined EC_{50} values are in good agreement with results of the fura-2 assay. However, compared with the binding experiments, the concentration–response curves of the agonists determined in the calcium assays were shifted 10- to 100-fold to the right. This was also described by Dautzenberg et al. (2005) for the hY_1, hY_4 and mY_5 receptors co-expressed with the chimeric G protein Gq_{i9} in HEK293 cells. Interestingly, only small differences between binding and functional data were observed at the hY_2 receptor co-expressed with the Gq_{i9} protein (Dautzenberg et al., 2005). Presumably, the extent of the rightward shift depends on the different coupling efficiency of the hY_2 receptor with the different chimeric G proteins. As the fluorescence-labeled porcine neuropeptide Y is a receptor agonist at the hY_2 receptor (not shown), the simultaneous determination of activity and affinity in a flow cytometric assay became feasible (Schneider, 2005; Schneider et al., 2006).

After stable transfection of the CHO-hY_2-Gq_{i5} cells with the apoaequorin gene targeted to the mitochondria an aequorin assay in the 96-well format was established. The reconstitution of active aequorin can be performed with cells in suspension (Dupriez et al., 2002) as this procedure is more convenient and produces less consumable cost compared to the reconstitution in adherent growing cells. The Tecan GeniosPro microplate reader is well suited for the aequorin assay as the instrument allows the immediate recording of flash luminescence after two separate injections. The aequorin luminescence gives a strong and robust signal which is stable for at least 2 h. The consumption of remaining active aequorin caused by cell lysis due to the injection of Triton-X-100 makes the measurement independent from variations of the number of injected cell during the assay. Receptor agonists as well as antagonists can be tested while the latter have to be preincubated with the cells prior to the injection of a fixed agonist concentration. The duration of the preincubation period with the receptor antagonist may influence the shape of the concentration–response curve of porcine neuropeptide Y as shown for BIIE0246 (Fig. 8). BIIE0246 behaved as an apparently competitive surmountable receptor antagonist when co-applied with the agonist, whereas a rightward shift of the concentration–response curve with a concomitant depression of the maximal effect is observed after preincubation of the cells in the presence of the receptor antagonist for 1 h. This is in agreement with a recent report by Dautzenberg and Neysari (2005) who found insurmountable antagonism after preincubation with the antagonist using HEK293 cells stably expressing the hY_2 receptor and the chimeric G protein Gq_{i9} in a fluorescence-based calcium assay.

In summary, the established flow cytometric binding assays using fluorescence-labeled porcine neuropeptide Y and stably transfected cells are an innovative alternative to neuropeptide Y receptor radioligand binding assays. After stable co-transfection of CHO cells with hY_2 receptors and the chimeric Gq_{i5} subunit, redirecting Y_2 receptor coupling to the phosphoinositide pathway, the CHO-hY_2-Gq_{i5} cells can be used for calcium assays using fluorescent indicators. Additional stable transfection of the cells with the apoaequorin gene targeted to the mitochondrial matrix converts the calcium signal into a luminescent signal. By exploitation of this phenomenon a luminescence-based assay could be established in 96-well plates. These techniques may also be useful for the investigation of other G protein-coupled receptors, provided that appropriate fluorescent probes and genetically engineered cells are available.

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References

Abbott, C.R., Small, C.J., Kennedy, A.R., Neary, N.M., Sajedi, A., Ghatei, M.A., Bloom, S.R., 2005. Blockade of the neuropeptide Y Y2 receptor with the specific antagonist BIIE0246 attenuates the effect of endogenous and exogenous peptide YY(3–36) on food intake. Brain Res. 1043, 139–144.

Batterham, R.L., Cowley, M.A., Small, C.J., Herzog, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A., Cone, R.D., Bloom, S.R., 2002. Gut hormone PYY(3–36) physiologically inhibits food intake. Nature 418, 650–654.

Beck-Sickinger, A.G., Grouzmann, E., Hoffmann, E., Gaida, W., van Meir, E.G., Waeber, B., Jung, G., 1992. A novel cyclic analog of neuropeptide Y specific for the Y2 receptor. Eur. J. Biochem. 206, 957–964.

Blinks, J.R., 1978. Applications of calcium-sensitive photoproteins in experimental biology. Photochem. Photobiol. 27, 423–432.

Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., Rizzuto, R., 1995. Transfected aequorin in the measurement of cytosolic Ca2+ concentration ([Ca2+]c). A critical evaluation. J. Biol. Chem. 270, 9896–9903.

Button, D., Brownstein, M., 1993. Aequorin-expressing mammalian cell lines used to report Ca2+ mobilization. Cell Calcium 14, 663–671.

Cabrele, C., Wieland, H.A., Langer, M., Stidsen, C.E., Beck-Sickinger, A.G., 2001. Y-receptor affinity modulation by the design of pancreatic polypeptide/neuropeptide Y chimera led to Y(5)-receptor ligands with picomolar affinity. Peptides 22, 365–378.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Coward, P., Chan, S.D.H., Wada, H.G., Humphries, G.M., Conklin, B.R., 1999. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal. Biochem. 270, 242–248.

Dautzenberg, F.M., 2005. Stimulation of neuropeptide Y-mediated calcium responses in human SMS-KAN neuroblastoma cells endogenously expressing Y2 receptors by co-expression of chimeric G proteins. Biochem. Pharmacol. 69, 1493–1499.

Dautzenberg, F.M., Neysari, S., 2005. Irreversible binding kinetics of neuropeptide Y ligands to Y2 but not to Y1 and Y5 receptors. Pharmacology 75, 21–29.

Dautzenberg, F.M., Higelin, J., Pflieger, P., Neidhart, W., Guba, W., 2005. Establishment of robust functional assays for the characterization of neuropeptide Y (NPY) receptors: identification of 3-(5-benzoyl-thiazol-2-ylamino)-benzonitrile as selective NPY type 5 receptor antagonist. Neuropharmacology 48, 1043–1055.

Dollinger, H., Esser, F., Mihm, G., Rudolf, K., Schnorrenberg, G., Gaida, W., Doods, H.N., 1999. Preparation of novel peptides for use as NPY antagonists. Boehringer Ingelheim Pharma, Ger. Offen. DE 19816929. Chem. Abstr. 131, 286832.

Doods, H., Gaida, W., Wieland, H.A., Dollinger, H., Schnorrenberg, G., Esser, F., Engel, W., Eberlein, W., Rudolf, K., 1999. BIIE0246: a selective and high affinity neuropeptide Y Y2 receptor antagonist. Eur. J. Pharmacol. 384, R3–R5.

Dumont, Y., Gaudreau, P., Mazzuferi, M., Langlois, D., Chabot, J.G., Fournier, A., Simonato, M., Quirion, R., 2005. BODIPY-conjugated neuropeptide Y ligands: new fluorescent tools to tag Y1, Y2, Y4 and Y5 receptor subtypes. Br. J. Pharmacol. 146, 1069–1081.

Dupriez, V.J., Maes, K., Le Poul, E., Burgeon, E., Detheux, M., 2002. Aequorin-based functional assays for G-protein-coupled receptors, ion channels, and tyrosine kinase receptors. Recept. Channels 8, 319–330.

Edwards, B.S., Oprea, T., Prossnitz, E.R., Sklar, L.A., 2004. Flow cytometry for high-throughput, high-content screening. Curr. Opin. Chem. Biol. 8, 392–398.

Edwards, B.S., Bologa, C., Young, S.M., Balakin, K.V., Prossnitz, E.R., Savchuck, N.P., Sklar, L.A., Oprea, T.I., 2005. Integration of virtual screening with high-throughput flow cytometry to identify novel small molecule formylpeptide receptor antagonists. Mol. Pharmacol. 68, 1301–1310.

Gerald, C., Walker, M.W., Vaysse, P.J.-J., He, C., Branchek, T.A., Weinshank, R.L., 1995. Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. J. Biol. Chem. 270, 26758–26761.

Goumain, M., Voisin, T., Lorinet, A.-M., Ducroc, R., Tsocas, A., Roze, C., Rouet-Benzineb, P., Herzog, H., Balasubramaniam, A., Laburthe, M., 2001. The peptide YY-preferring receptor mediating inhibition of small intestinal secretion is a peripheral Y2 receptor: pharmacological evidence and molecular cloning. Mol. Pharmacol. 60, 124–134.

Hatse, S., Princen, K., Liekens, S., Vermeire, K., De Clercq, E., Schols, D., 2004. Fluorescent CXCL12AF647 as a novel probe for nonradioactive CXCL12/CXCR4 cellular interaction studies. Cytometry A 61, 178–188.

Hazelwood, R.L., 1993. The pancreatic polypeptide (PP-fold) family: gastrointestinal, vascular, and feeding behavioral implications. Proc. Soc. Exp. Biol. Med. 202, 44–63.

Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T., Tsuji, F.I., 1985. Cloning and sequence analysis of cDNA for the luminescent protein aequorin. Proc. Natl. Acad. Sci. U. S. A. 82, 3154–3158.

Le Poul, E., Hisada, S., Mizuguchi, Y., Dupriez, V.J., Burgeon, E., Detheux, M., 2002. Adaptation of aequorin functional assay to high throughput screening. J. Biomol. Screen. 7, 57–65.

Lynch, J.W., Lemos, V.S., Bucher, B., Stoclet, J.C., Takeda, K., 1994. A pertussis toxin-insensitive calcium influx mediated by neuropeptide Y2 receptors in a human neuroblastoma cell line. J. Biol. Chem. 269, 8226–8233.

Michel, M.C. (Ed.), 2004. Neuropeptide Y and Related Peptides, Handbook of Experimental Pharmacology, vol. 162. Springer, Berlin.

Michel, M.C., Beck-Sickinger, A., Cox, H., Doods, H.N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., Westfall, T., 1998. XVI. International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. Pharmacol. Rev. 50, 143–150.

Müller, M., Knieps, S., Gessele, K., Dove, S., Bernhardt, G., Buschauer, A., 1997. Synthesis and neuropeptide Y Y1 receptor antagonistic activity of *N*, *N*-disubstituted omega-guanidino- and omega-aminoalkanoic acid amides. Arch. Pharm. (Weinh.) 330, 333–342.

Neubig, R.R., Spedding, M., Kenakin, T., Christopoulos, A., 2003. International union of pharmacology committee on receptor nomenclature and drug classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol. Rev. 55, 597–606.

Rose, P.M., Fernandes, P., Lynch, J.S., Frazier, S.T., Fisher, S.M., Kodukula, K., Kienzle, B., Seethala, R., 1995. Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor. J. Biol. Chem. 270, 22661–22664.

Schaeffer, M.T., Cully, D., Chou, M., Liu, J., Van der Ploeg, L.H., Fong, T.M., 1999. Use of bioluminescent aequorin for the pharmacological characterization of 5HT receptors. J. Recept. Signal Transduct. Res. 19, 927–938.

Schneider, E., 2005. Development of Fluorescence-Based Methods for the Determination of Ligand Affinity, Selectivity and Activity at G-Protein Coupled Receptors. Doctoral thesis, University of Regensburg.

Schneider, E.S., Mayer, M., Ziemek, R., Li, L., Hutzler, C., Bernhardt, G., Buschauer, A., 2006. A Simple and Powerful Flow Cytometric Method for the Simultaneous Determination of Multiple Parameters at G-Protein-coupled Receptor Subtypes. ChemBioChem 7, 1400–1409.

Stables, J., Green, A., Marshall, F., Fraser, N., Knight, E., Sautel, M., Milligan, G., Lee, M., Rees, S., 1997. A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. Anal. Biochem. 252, 115–126.

Stables, J., Mattheakis, L.C., Chang, R., Rees, S., 2000. Recombinant aequorin as reporter of changes in intracellular calcium in mammalian cells. Methods Enzymol. 327, 456–471.

Stein, R.A., Wilkinson, J.C., Guyer, C.A., Staros, J.V., 2001. An analytical approach to the measurement of equilibrium binding constants: application to EGF binding to EGF receptors in intact cells measured by flow cytometry. Biochemistry 40, 6142–6154.

Torfs, H., Poels, J., Detheux, M., Dupriez, V., Van Loy, T., Vercammen, L., Vassart, G., Parmentier, M., Vanden Broeck, J., 2002. Recombinant aequorin as a reporter for receptor-mediated changes of intracellular Ca2+-levels in Drosophila S2 cells. Invertebr. Neurosci. 4, 119–124.

Ungrin, M.D., Singh, L.M., Stocco, R., Sas, D.E., Abramovitz, M., 1999. An automated aequorin luminescence-based functional calcium assay for G-protein-coupled receptors. Anal. Biochem. 272, 34–42.