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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

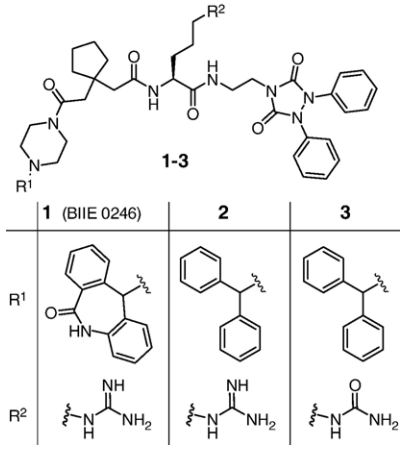

Fig. 1. Structural formulas of Y_2 receptor antagonists.