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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

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Abstract

The human Neuropeptide Y (NPY) receptors 1 (hY_1), 2 (hY_2), 4 (hY_4), and the mouse type 5 (mY_5) receptor were expressed in human embryonic kidney 293 (HEK293) cells. The receptors bound a radioiodinated NPY ligand with high affinity and various NPY analogs competed for binding in a receptor selective-manner. Similarly, cAMP-inhibition and GTPγS binding assays were established. The four NPY receptors were further tested in the fluorimetric imaging plate reader (FLIPR) format, a cellular high-throughput assay, in the absence and presence of chimeric G proteins, Gq_{o5}, Gq_{i5} and Gq_{i9}. The receptors stimulated transient calcium release only in the presence of chimeric G proteins. While hY_1, hY_2 and hY_4 receptors coupled to Gq_{o5}, Gq_{i5} and Gq_{i9}, the mY_5 receptor stimulated transient calcium release only when co-expressed with Gq_{i9}. Using an in silico screening approach we identified a small molecule 3-(5-benzoyl-thiazol-2-ylamino)-benzonitrile (compound **1**), which bound to the mY_5 receptor with high affinity ($K_i = 32.1 \pm 1.8$ nM), competitively antagonized NPY-mediated GTPγS binding and calcium stimulation with high potency, and had no affinity for other NPY receptors. These data show that NPY receptors can be functionally coupled to the FLIPR readout, allowing for high throughput compound testing and identification of novel molecules.

Keywords: Neuropeptide Y receptor; Ligand binding; Cyclic AMP inhibition; GTPγS binding; Fluorimetric imaging plate reader; Antagonism

1. Introduction

The 36-amino-acid neuropeptide Y (NPY) is the most abundant peptide in the brain (Gehlert, 1999; Wieland et al., 2000; Holmes et al., 2003). Besides NPY two highly related peptides, pancreatic polypeptide (PP) and peptide YY (PYY) have been isolated (Gehlert, 1999; Wieland et al., 2000; Kask et al., 2002). NPY, PYY and PP have been implicated in the development of a variety of peripheral and central disorders including

Abbreviations: NPY, Neuropeptide Y; PYY, Peptide YY; PP, Pancreatic Polypeptide; Y, NPY receptor; FLIPR, fluorimetric image plate reader; GPCR, G protein-coupled receptor; $G_{i/o}$, inhibitory GTP binding protein; cAMP, cyclic AMP; h, human; m, mouse; p, porcine.

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cardiovascular and respiratory disorders, intestinal dysfunction, type II diabetes, anxiety and depression, epilepsy and feeding disorders (Gehlert, 1999; Kask et al., 2002; Balasubramaniam, 2003; Holmes et al., 2003; Levens and Della-Zuana, 2003). NPY and related peptides are the most potent orexigenic stimuli (Gehlert, 1999; Balasubramaniam, 2003; Levens and Della-Zuana, 2003). Acute or chronic central application of NPY stimulates robust feeding behavior and substantial weight gain associated with increased fat mass (Gehlert, 1999; Iyengar et al., 1999).

NPY peptides mediate their activity through at least five different NPY receptors, Y_1, Y_2, Y_4, Y_5 and y_6, the latter of which only exists in mouse and rabbit (Blomqvist and Herzog, 1997; Michel et al., 1998). Although NPY, PYY and PP are highly homologous on amino acid level, NPY receptors are only distantly related and to some extent show as little as 30% homology amongst each other (Blomqvist and Herzog, 1997; Wraith et al., 2000). All NPY receptors belong to the large superfamily of G protein-coupled receptors (GPCR) and are coupled to the inhibitory G protein $G_{i/o}$, which upon receptor activation reduces intracellular cyclic AMP (cAMP) levels (Wieland et al., 2000). NPY receptors display distinct binding and pharmacological profiles (Michel et al., 1998; Wieland et al., 2000). The Y_1 receptor binds NPY and PYY with high affinity whereas PP and N-terminally truncated analogs are poor ligands. The first selective Y_1 antagonist that has been reported is BIBP3226 (Rudolf et al., 1994). The Y_2 receptor preferentially binds NPY and PYY over PP but is very insensitive to N-terminal NPY-analog truncations. A C-terminal cyclic NPY dodecapeptide, cyclo[K^{28}-E^{32}]NPYAc_{25-36} is a highly selective and potent Y_2 receptor agonist (Rist et al., 1996). Recently, BIIE0246 has been reported as selective Y_2 receptor antagonist (Doods et al., 1999). The Y_4 receptor, in contrast to all other NPY receptors preferentially binds PP although the human Y_4 receptor (hY_4) also binds NPY, PYY and N-terminally truncated NPY analogs with nanomolar affinity (Michel et al., 1998; Wieland et al., 2000). No small molecule Y_4 receptor antagonists thus far have been reported (Balasubramaniam, 2003). Finally, the Y_5 receptor does not discriminate between NPY and PYY, binds PP with only micromolar affinity, and is relatively insensitive to N-terminal truncations (Michel et al., 1998; Wieland et al., 2000). Recently, a large number of small molecule antagonists targeting Y_5 have been disclosed in patent literature and scientific publications (Levens and Della-Zuana, 2003). One example is L-152,804, an orally active selective Y_5 receptor antagonist (Kanatani et al., 2000).

As mentioned above, NPY is the most potent orexigenic peptide known to date. Because obesity has become an epidemic over the past years, academic and pharmaceutical research has been intensified to develop new anti-obesity drugs, and NPY receptors have become attractive drug targets. While Y_1 receptors have initially been subject to intensive research, activities have been shifted to the Y_5 receptor, which has a limited expression profile in the human brain (Blomqvist and Herzog, 1997) and thus appears to be a safer target for the treatment of obesity. Recently, genetic manipulations and human studies with a Y_2 receptor-preferring agonist, PYY_{3-36} also directed attention towards this receptor as a target for anti-obesity research (Naveilhan et al., 1999; Batterham et al., 2002).

In our continuous attempts to develop new drugs for the treatment of obesity we established a program for the development of Y_2-selective agonists and Y_5-selective antagonists. Because NPY receptors are coupled to the inhibition of cAMP we aimed at the development of robust functional assays instead of the time consuming and rather low throughput cAMP inhibition assays. We report on the establishment of GTPγS binding assays and the robust transient calcium (Ca^{2+}) mobilization assay in the fluorimetric imaging plate reader (FLIPR) format. Finally, we present data on the rapid identification of a highly selective and potent Y_5 receptor antagonist from the Roche compound library using a virtual screening approach.

2. Materials and methods

2.1. Materials, peptides, reagents and radiochemicals

Cell culture media and reagents were purchased from Gibco/BRL. The NPY peptides (purity >95%) were obtained from Bachem Corp. (Bubendorf, Switzerland), cyclo[K^{28}-E^{32}] NPYAc25–36 (purity >95%) as well as BIBP-3226 and BIEE-0246 (purity >98%) were kindly provided by Prof. Dr. Annette Beck-Sickinger, University of Leipzig, Germany. L-152,804 (purity >95%) was obtained from Tocris (Avonmouth, UK). [^{125}I]PYY (2000 Ci/mmol) of porcine origin and GTPγ^{35}S (1130 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, UK).

2.2. Molecular cloning

Total RNA from mouse amygdala and human hypothalamus was isolated using the RNeasy kit (Qiagen, Hilden, Germany) and first-strand cDNA was synthesized as described (Dautzenberg et al., 1997). Full-length hY_1, hY_2, hY_4 and mouse Y_5 (mY_5) cDNAs were cloned by PCR using the primer combinations Y_{1forw} (5′-AATGAATTCAACATTATTTTCCCAGG-3′, nucleotides 150–176 of hY_1, accession no. A26481) and hY_{1rev} (5′-GGGACCATAGGCTATAAGTAGTTTC-3′, nucleotides 1329–1304 of hY_1), hY_{2forw} (5′-TACTGAA AATGGGTCCAATAGGTG-3′, nucleotides 9–36 of