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Stimulation of neuropeptide Y-mediated calcium responses in human SMS-KAN neuroblastoma cells endogenously expressing Y_2 receptors by co-expression of chimeric G proteins

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Abstract

Human SMS-KAN neuroblastoma cells endogenously express the neuropeptide Y (NPY) type 2 (Y_2) receptor. Although ligand binding and GTPγS binding studies supported high functional Y_2 receptor expression, only weak coupling to the natural second messenger cyclic AMP was observed. The main reason was the low responsiveness of SMS-KAN cells to forskolin, a direct activator of adenylyl cyclases. In order to obtain a cell-based functional assay for the Y_2 receptor in SMS-KAN cells, the transient calcium (Ca^{2+}) mobilization assay in the fluorimetric imaging plate reader (FLIPR) format was established by stably expressing a chimeric G protein Gq_{i9}. This manipulation resulted in robust mobilization of Ca^{2+} after challenge with various NPY-related agonists in a 384-well format. The sensitivity of the FLIPR readout was in the low nanomolar range for NPY agonists and comparable to that of the recombinant Y_2 receptor. The selective Y_2 antagonist BIIE0246 competitively inhibited NPY-mediated Ca^{2+} transients in SMS-KAN/Gq_{i9} cells with a pA_2 value of 7.39 ± 0.1. This is the first evidence that an endogenously expressed G protein-coupled receptor couples to an overexpressed chimeric G protein, thereby functionally responding in the FLIPR readout.

Keywords: Ligand binding; cAMP inhibition; GTPγS binding; Ca^{2+} stimulation; Chimeric G protein

1. Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide that belongs to a peptide family further comprising peptide YY (PYY) and pancreatic polypeptide (PP), is widely distributed in the central and peripheral nervous system [1,2]. In the brain, NPY is the most abundant neuropeptide known to date. Its localization argues in favor of an involvement in various physiological processes including anxiety, learning and memory, circadian rhythm, water and food intake [1,3–5]. Moreover, NPY and related peptides are the most potent orexigenic stimuli known to date [1,4,5]. Acute or chronic central application of NPY stimulates robust feeding behavior and substantial weight gain associated with increased fat mass [1,6].

NPY peptides bind to and activate at least five different NPY receptors, Y_1, Y_2, Y_4, Y_5 and y_6 [7,8]. All NPY receptors belong to the large superfamily of G protein-coupled receptors (GPCR) and are coupled to the pertussis-toxin (PTX) sensitive inhibitory G protein $G_{i/o}$, which reduces intracellular cyclic AMP (cAMP) levels [2]. In the mammalian brain Y_1 and Y_2 receptors are the most abundant NPY receptors [9]. While the Y_1 receptor is discretely located in the cortex and thalamus, Y_2 mRNA is evenly distributed in several brain areas that are implicated for the development of psychiatric and eating disorders [1,4,9–11]. The recent findings that Y_2-deficient animals develop obesity and the anti-obesity effects of $PYY_{3–36}$, a rather selective Y_2 receptor agonist, in rodents and humans [12–14] attracted research into the development

Abbreviations: NPY, neuropeptide Y; $Y_{1–5}$, NPY type 1–5 receptor; y_6, NPY type 6 receptor; GPCR, G protein-coupled receptor; G_o and G_i protein, inhibitory G proteins that are negatively coupled to adenylate cyclase; Gq protein, phosphoinositide- and calcium stimulating G protein; Gq_{o5}; Gq_{i5} and Gq_{i9}, chimeric G proteins carrying the last 5–9 amino acids of G_o and G_i proteins linked to the Gq protein

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of Y_2 receptor-selective agonists for the treatment of eating disorders.

A common problem in pharmaceutical research is the fact that many targets are subject to intellectual property claims, which block companies from using recombinant material [15]. For the Y_2 receptor, there are at least three granted patents, which hamper usage of the recombinant receptor [16–18]. Consequently, my laboratory got interested in identifying a cell line endogenously expressing the human Y_2 receptor and which can be easily adapted to robust functional assays with intact cells. The human neuroblastoma line SMS-KAN has been reported to express high levels of Y_2 receptors [19,20] but despite sufficient binding data on the SMS-KAN Y_2 receptor there is little data on functional responses in this cell line.

In this study, several functional assays including cAMP, GTPγS binding and transient calcium (Ca^{2+}) mobilization assays were tested for application to the Y_2 receptor in SMS-KAN cells. Data will be presented showing that by co-transfection of a chimeric G protein the endogenous Y_2 receptors robustly responded in the high-throughput fluorimetric imaging plate reader (FLIPR) format allowing for rapid screening based on transient Ca^{2+} mobilization.

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 Corporation (Bubendorf, Switzerland), cyclo[K^{28}-E^{32}] $NPY_{AC25–36}$ (purity >95%) and BIIE0246 (purity >98%) were kindly provided by Prof. Dr. Annette Beck-Sickinger, University Leipzig, Germany. Pertussis toxin (PTX) was from Calbiochem (Bad Soden, Germany). ^{125}I-PYY (2000 Ci/mmol) of porcine origin and GTPγ^{35}S (1130 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, UK). The cDNAs encoding chimeric G proteins Gq_{o5}, Gq_{i5} and Gq_{i9} in pcDNA3 (Invitrogen, La Jolla, CA) were kindly provided by Dr. Pari Malherbe, F. Hoffmann-La Roche AG, Basel, Switzerland.

2.2. Cell transfections

Initially, cDNAs coding for Gq_{o5}, Gq_{i5} and Gq_{i9} or the empty pcDNA3 vector (20 μg each) were transiently transfected into SMS-KAN cells with the FUGENE reagent (Roche Molecular Biochemicals, Mannheim, Gemany) according to the manufacturer's protocol. Two days after transfection cells were tested for their responsiveness in the FLIPR format. Cell lines stably expressing Gq_{i9} were generated by transfecting the cDNA (1 μg) with the FUGENE reagent. Two days after transfection, geniticin (500 μg/ml) selection was initiated and stable cell clones, robustly responding in the FLIPR assay were selected.

2.3. Radioreceptor binding assays

Membranes from SMS-KAN cells were prepared as described [11]. Competition binding assays were performed with 10 μg of membrane proteins prepared from SMS-KAN cells under assay conditions described in Rist et al. in 96-well plates (Beckmann Instruments, Fullertown, CA) using a scintillation proximity assay (SPA) [21]. The membranes, 0.5 mg wheatgerm agglutinin SPA beads (Amersham Pharmacia Biotech), 100 pM ^{125}I-PYY and unlabeled peptides (10^{-6} to 10^{-11} M) were added. The reaction mixture was incubated on a shaker for 120 min at 22 °C and then read in a TopCount (Packard). Non-specific binding was determined as residual binding in the presence of 10 μM PYY. The inhibition constant, K_i was calculated using the interactive curve fitting program Xlfit (IDBS, Guilford, UK). Under these conditions less than 10% of the total radioactivity was specifically bound by SMS-KAN membranes.

2.4. GTPγS binding assay

Agonist-mediated binding of GTPγ^{35}S was investigated in 96-well plates by SPA format [22] using membranes prepared from SMS-KAM cells. Binding was performed in 200 μl 20 mM HEPES-buffer (pH 7.4, plus 6 mM $MgCl_2$ and 100 mM NaCl), supplemented with 20 μM GDP, 10 μM cold GTPγS and 0.3 nM GTPγ^{35}S. Twenty micrograms membranes, 1 mg wheatgerm agglutinin SPA beads, NPY analogs or synthetic compounds (10^{-5}–10^{-10} M) were added. The reaction mixture was incubated on a shaker for 60 min at 22 °C and then centrifuged for 5 min at 1500 rpm in an Eppendorf 5403 centrifuge. Finally the plates were read in a TopCount reader (Packard).

2.5. cAMP-inhibition assay

Forskolin-mediated cAMP accumulation in SMS-KAN cells was determined in 96-well plates as previously described [23]. Briefly 50,000 cells were incubated in Krebs–Ringer-HEPES-buffered solution (KHR; 124 mM NaCl, 5 mM KCl, 1.25 mM $MgSO_4$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, pH 7.4), supplemented with 1 mM 3-isobutyl-1-methylxanthine (IBMX) in the presence of increasing concentrations (1 to 50 μM) forskolin (Sigma, Munich, Germany) for 60 min at 37 °C. Reactions were stopped by the addition of 0.12 ml ice-cold ethanol and stored at −80 °C for at least 4 h. The cAMP content was determined from the supernatant using the Biotrak non-radioactive cAMP kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.6. Calcium mobilization assays

SMS-KAN cells transiently or stably expressing chimeric G proteins were seeded at a density of 100,000 cells

into poly-D-lysine coated 384-well blackwall, clear-bottom microtiter plates (Corning, NY) as described previously [24]. HEK293 cells transiently transfected with human Y_2 cDNA with or without chimeric G proteins were treated in a similar manner, except that only 20,000 cells were used. One day later, the medium was removed and 50 μl loading medium [DMEM high glucose, without serum, supplemented with 10 mM HEPES-acid, 0.1% BSA, 5 mM probenecid and 2 μM Fluo-3AM (Molecular Probes, Leiden, The Netherlands)] was added. Cells were loaded for 1 h at 37 °C, washed twice with 50 μl assay buffer (5 mM HEPES-acid, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 10 mM glucose) and then 30 μl assay buffer was added. Cells were further pre-incubated at room temperature before adding agonists or agonists plus antagonists in 20 μl assay buffer and then measured on a T-channel fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). Maximum change in fluorescence over baseline was used to determine agonist response.

2.7. Statistical analyses

Statistical analyses of the binding, cAMP, GTPγS and calcium mobilization data were performed on a MacIntosh PC using StatView software (SAS, Cary, NC) by analyses of one-way variance (ANOVAs) across experimental groups followed by the Dunnett's test with the alpha set at 0.05.

3. Results

3.1. Binding of various NPY ligands to membranes from SMS-KAN cells

Membranes from SMS-KAN cells bound ^{125}I-PYY with high affinity ($K_d = 0.08 \pm 0.01$ nM) and high receptor numbers ($B_{max} = 0.8 \pm 0.15$ pmol/mg protein) (not shown). Thus, for competition binding experiments ~100 pM radiolabel and as little as 10 μg membrane proteins were applied in the SPA format. In the competition binding experiments PYY ($K_i = 0.36 \pm 0.08$ nM), cyclo[K^{28}–E^{32}]$NPY_{AC25–36}$ ($K_i = 1.94 \pm 0.22$ nM), NPY ($K_i = 2.12 \pm 0.44$ nM), $NPY_{13–36}$ ($K_i = 11.2 \pm 1.9$ nM) and BIIE0246 ($K_i = 36.1 \pm 4.8$ nM) bound to SMS-KAN membranes with the typical Y_2 receptor pharmacology (Fig. 1), whereas PP, a Y_4-preferring ligand at concentrations up to 1 μM did not compete for ^{125}I-PYY binding (not shown).

3.2. GTPγS binding

When membranes from SMS-KAN cells were tested for their capability to bind GTPγ^{35}S after NPY-agonist chal-

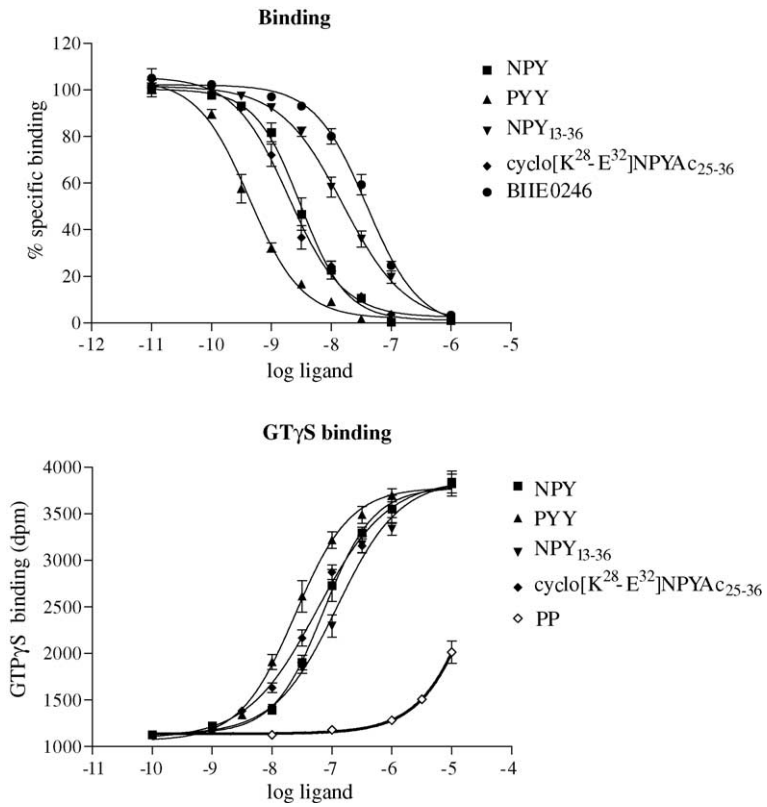

Fig. 1. Concentration-dependent inhibition of ^{125}I-PYY binding and NPY agonist-mediated GTPγ^{35}S binding to SMS-KAN membranes. The membranes (10–20 μg) were incubated at room temperature for 60 min with increasing concentrations of NPY agonists as described in Section 2. The results are representative of four independent experiments performed in triplicate.

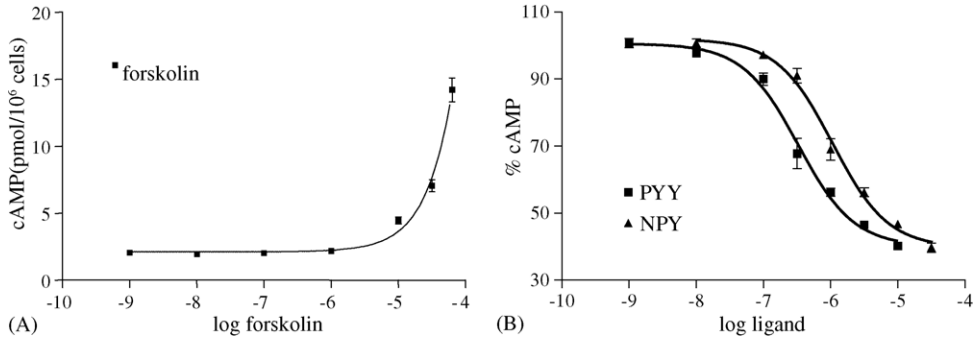

Fig. 2. Stimulation of cAMP production in SMS-KAN cells by forskolin (A) and inhibition of forskolin-mediated cAMP production by PYY and NPY (B); (A) SMS-KAN cells (50,000 cells/well) were stimulated in a 96-well format with increasing concentrations of forskolin for 60 min at 37 °C; (B) increasing concentrations (1 nM to 30 μM) of PYY or NPY were co-incubated with 50 μM forskolin for 60 min at 37 °C. The results are representative of three independent experiments performed in quadruplicate.

lenge, robust specific binding, ~3.5-fold over basal levels, were measured, indicating functional coupling of the endogenous Y_2 receptor (Fig. 1). A similar potency rank order profile as observed for the binding profile was obtained for GTPγS binding (Fig. 1): PYY ($EC_{50} = 22.6 \pm 3.1$ nM) > cyclo [K^{28}-E^{32}]$NPY_{AC25-36}$ ($EC_{50} = 53.1 \pm 6.4$ nM) ≥ NPY ($EC_{50} = 76.4 \pm 8.2$ nM) > NPY_{13-36} ($EC_{50} = 126 \pm 12$ nM) ≫ PP ($EC_{50} = 5.4 \pm 1.6$ μM).

3.3. Forskolin-mediated cAMP accumulation in SMS-KAN cells

Initially, only small increases (~2–3-fold) of intracellular cAMP levels were measured when SMS-KAN cells were stimulated with 10 μM forskolin (not shown). Therefore, a concentration-response curve was generated for forskolin and SMS-KAN cells. Concentrations from as little as 1 nM to the maximally applicable concentration of 50 μM forskolin were used to stimulate cAMP production in SMS-KAN cells. Concentrations up to 1 μM forskolin did not stimulate intracellular cAMP levels over basal values (Fig. 2A) and only 2-fold (10 μM), 3.5-fold (20 μM) and 7-fold (50 μM) elevations were observed at very high forskolin concentrations (Fig. 2A). However, no plateau was observed. Although, these results indicated that there might be a deficit in the forskolin-responsiveness, the potency of PYY and NPY to inhibit forskolin-mediated (50 μM) cAMP production was tested. PYY ($EC_{50} = 356 \pm 44$ nM) and NPY ($EC_{50} = 1.21 \pm 0.09$ μM) mM) inhibited forskolin responses by ~60% only at very high agonist concentrations (Fig. 2B).

3.4. Transient Ca^{2+} mobilization in SMS-KAN cells

SMS-KAN cells were tested for their capability to transiently stimulate NPY-mediated Ca^{2+} mobilization in the FLIPR format. Because GTPγS binding and cAMP-inhibition experiments indicated a coupling to $G\alpha_{i/o}$ proteins, cells were transiently transfected with three chimeric G proteins, Gq_{o5}, Gq_{i5} and Gq_{i9} or an empty vector serving as control. As exemplified in Fig. 3, 1 μM PYY stimulated Ca^{2+} transients ~3-fold over basal levels in Gq_{i9}-transfected SMS-KAN cells but not in cells transiently transfected with the empty vector control. Like Gq_{i9}, Gq_{o5} and Gq_{i5} also stimulated transient Ca^{2+} mobilization ~3-fold over basal levels in SMS-KAN cells (Fig. 4). Although much higher stimulation values were obtained for HEK293 cells recombinantly expressing the human Y_2 receptor (Fig. 4), the values obtained with the SMS-KAN cells were considered robust enough for further profiling NPY agonists and the Y_2-selective antagonist BIIE0246.

Concentration-response curves for PYY, NPY, NPY_{3-36}, NPY_{13-36} and cyclo[K^{28}–E^{32}]$NPY_{AC25-36}$ were generated in SMS-KAN cells stably expressing the Gq_{i9} protein. These agonists stimulated Ca^{2+} transients with potencies in the low nanomolar range (Fig. 5, Table 1). When increasing concentrations of BIIE0246 were assessed for their potency to inhibit cyclo[K^{28}-E^{32}]$NPY_{AC25-36}$-stimulated (20 nM) Ca^{2+} transients, Schild regressions revealed a pA_2 value of 7.39 ± 0.1, which is in good agreement with its binding affinity to SMS-KAN Y_2 receptors.

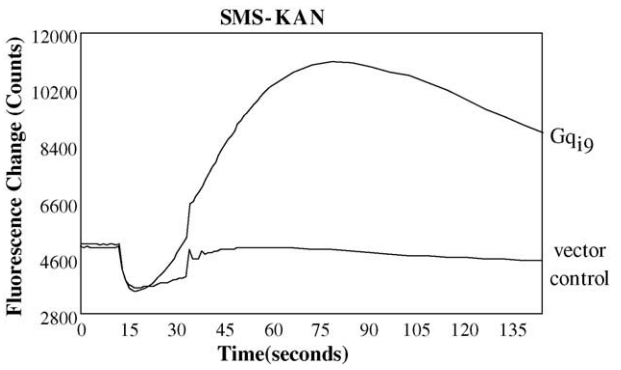

Fig. 3. Real time transient Ca^{2+} mobilization responses in SMS-KAN cells transiently transfected with and empty vector or the chimeric G protein Gq_{i9} mediated by 1 μM PYY. SMS-KAN cells were transiently transfected. Two days after transfection cells were tested for their ability to elicit transient Ca^{2+} mobilization upon challenge with a 1 μM PYY concentration. Data are representative of three independent experiments performed in quadruplicate.

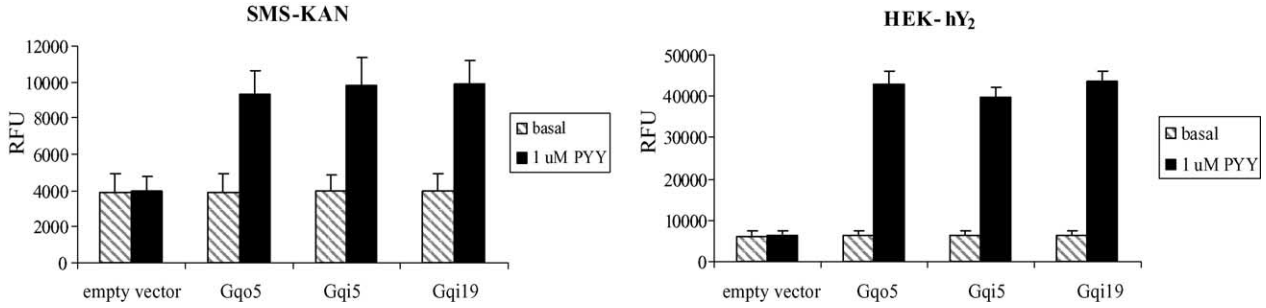

Fig. 4. Quantification of maximal PYY-mediated Ca^{2+} transients in SMS-KAN cells or HEK293-Y_2 cells transiently transfected with an empty vector or various chimeric G proteins. Cells were transiently transfected with cDNA coding for three different chimeric G proteins Gq_{o5}, Gq_{i5} or Gq_{i9} or with an empty vector only. Two days after transfection cells were tested for their ability to elicit transient Ca^{2+} mobilization upon challenge with PYY (1 μM). Data are representative of three independent experiments performed in quadruplicate.

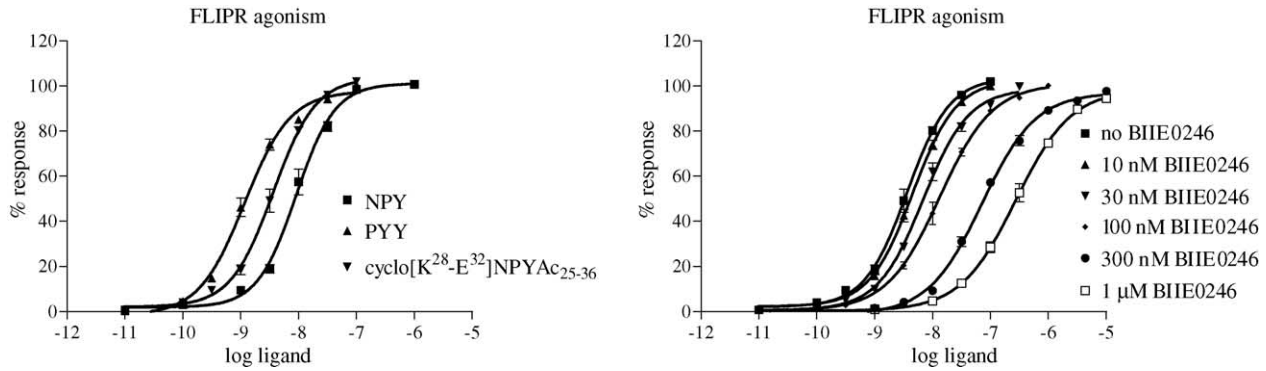

Fig. 5. Activation or inhibition of Ca^{2+} transients by various NPY agonists and the antagonists BIIE0246 in SMS-KAN cells stably expressing Gq_{i9}. NPY agonism: cells were stimulated with increasing concentrations of several NPY peptides under the conditions described in Section 2. Antagonism: various concentrations of the specific Y_2 antagonist BIIE0246 were incubated together with increasing concentrations of cyclo[K^{28}–E^{32}]$NPY_{AC25–36}$ as described in Section 2. Results are representative of five experiments performed in quadruplicate.

In a final experimental setting, the sensitivity of NPY-mediated FLIPR responses to pertussis toxin (PTX) was assessed. SMS-KAN cells were incubated for 16 h with PTX (25 ng/ml) [25] and then tested in the FLIPR and cAMP assays. In the cAMP assay SMS-KAN cells showed a high sensitivity to PTX treatment, in agreement with the predicted $G_{i/o}$-coupling of the Y_2 receptor (Fig. 6A). In contrast, SMS-KAN/Gq_{i9} cells remained insensitive to PTX treatment (Fig. 6B).

Table 1
Stimulation of transient Ca^{2+} mobilization by NPY agonists in SMS-KAN cells stably transfected with the Gq_{i9} protein

Agonist	EC_{50} (nM)	E_{max} (ΔRFU)
NPY	7.9 ± 1.4^{a}	5800 ± 350
PYY	1.4 ± 0.2	6100 ± 550
$NPY_{3–36}$	$9.4 \pm 1.3^{a,b}$	5700 ± 450
$NPY_{13–36}$	$12.6 \pm 2.4^{a,b}$	6000 ± 600
Cyclo[K^{28}–E^{32}]$NPY_{AC25–36}$	3.1 ± 0.4	6200 ± 200

Data are representative of five independent stimulations performed in quadruplicate. By ANOVA, there were significant differences across the groups for the EC_{50} values of Ca^{2+} mobilization ($F(4, 20) = 11.015$; $p < 0.0001$). The following additional post-hoc differences were found to be statistically significant: $^{a}p < 0.005$ vs. PYY; $^{b}p < 0.005$ vs. cyclo[K^{28}–E^{32}]$NPY_{AC25–36}$.

4. Discussion

In this study, a cellular high throughput screening assay for endogenously expressed NPY Y_2 receptors was established in human neuroblastoma SMS-KAN cells by co-expression of a chimeric G protein, Gq_{i9}.

The Y_2 receptor has become a highly interesting target for drug development because Y_2 receptor-deficient mice develop mild obesity [12]. In addition the Y_2 receptor preferring-agonist $PYY_{3–36}$ reduced appetite in humans [13,14] and inhibited food intake after either peripheral or central application in rodents [26]. Furthermore, in Y_2-deficient animals, $PYY_{3–36}$'s anorectic effect was abolished, indicating that $PYY_{3–36}$ mediates its anorectic effects exclusively through the Y_2 receptor [26]. While selective small molecule Y_2 antagonists have been published [11,27,28], no small molecule Y_2-selective agonists are known to date.

The development of Y_2-selective agonists however, requires potent and robust functional screening assays. These assays are normally run in recombinant receptor expression systems. Recently, the pharmaceutical industry is facing a serious problem: a large number of granted patents prohibiting usage of recombinant material for drug development [15]. For the recombinant Y_2 receptor, at least

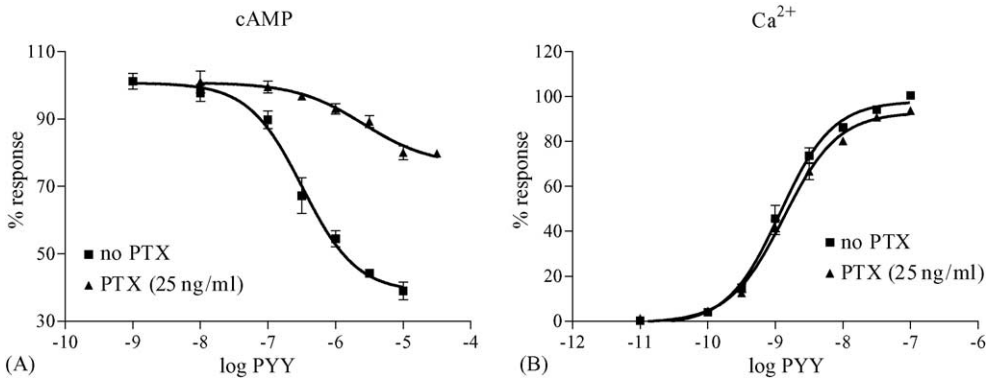

Fig. 6. Effects of PTX on cAMP (A) or FLIPR (B) responses in SMS-KAN (A) and SMS-KAN/Gq_{i9} (B) cells. Cells were incubated with PTX (25 ng/ml) for 16 h and then tested for their ability to promote PYY-mediated inhibition of forskolin-stimulated cAMP formation (A) or to stimulate Ca^{2+} transients (B) in comparison to control cells treated with vehicle. Results are representative of three experiments performed in quadruplicate.

three patents have been granted in Europe or the USA [16–18]. In order to develop an alternative approach, my laboratory was seeking a cell line endogenously expressing human Y_2 receptors to establish a robust functional and cellular assay. The SMS-KAN neuroblastoma line was chosen because it is a well documented tool for Y_2 receptor studies [11,19,20,29,30].

Radioligand binding and functional GTPγS binding experiments using SMS-KAN membranes revealed robust responses. NPY analogs were bound with the classical Y_2-specific profile [7,19] and these peptides also stimulated GTPγS binding with a similar potency rank order, in agreement with previous publications by other laboratories [11,19,20]. However, when NPY agonists were tested for their potency to inhibit forskolin-mediated cAMP production, it was noticed that forskolin was a poor activator of SMS-KAN cell adenylyl cyclases, and only highest practically applicable forskolin concentrations (~50 μM) stimulated cAMP production sufficiently. These results contrast with a publication by Shigeri and Fujimoto [31] who reported responsiveness of SMS-KAN cells to lower forskolin concentrations. A reason for this discrepancy could be that in different laboratories substrains of this cell line were generated, which respond differently in the cAMP assay. The deficient cAMP-signaling of the SMS-KAN cells became more evident when the potency of NPY agonists to inhibit cAMP production was assessed. At least 10-fold higher agonist concentrations than in the GTPγS binding experiments were needed to inhibit cAMP accumulation. This is in sharp contrast to results from my laboratory using recombinant Y_2 receptors [21].

Therefore, an alternative assay was chosen to functionally couple SMS-KAN cells in a cellular setting. My laboratory has previously reported the functional coupling of recombinant Y_1, Y_2, Y_4 and Y_5 receptors to the transient Ca^{2+} mobilization in the high-throughput FLIPR format [21] using a chimeric G protein approach [32]. This was applied for SMS-KAN cells. Like for recombinant Y_2 receptors [21], no functional coupling of the endogenous Y_2 receptor was observed in the FLIPR readout. However, after transient transfection of either Gq_{o5}, Gq_{i5} or Gq_{i9} proteins a three-fold elevation of transient Ca^{2+} mobilization over basal levels was recorded. In order to further compare SMS-KAN FLIPR responses with those of the recombinant system, a stable SMS-KAN/Gq_{i9} cell line was established and tested in a 384-well format. Various NPY agonists potently stimulated Ca^{2+} responses in this cell line at low nanomolar concentrations with the typical Y_2 profile [21]. Furthermore, BIIE0246 antagonized cyclo[K^{28}-E^{32}]$NPY_{AC25–36}$-stimulated FLIPR responses with a pA_2 value close to its competitive binding profile to the Y_2 receptor. Similar results were obtained with PYY or NPY as agonist (unpublished data). In contrast to cAMP-inhibition, FLIPR responses were completely insensitive to PTX treatment, indicating that indeed the Y_2 receptor coupling to the FLIPR readout was mediated by the PTX-insensitive chimeric G proteins [33].

In summary, SMS-KAN cells despite robustly binding NPY analogs and mediating NPY-stimulated GTPγS binding are not well coupled to cAMP inhibition. This deficiency is due to a low responsiveness to forskolin stimulation. However, by transfection of a chimeric G protein into SMS-KAN cells, the endogenous Y_2 receptor efficiently coupled to transient Ca^{2+} mobilization in the FLIPR readout even in a 384-well format and may serve as an alternative to standard recombinant systems. To my knowledge this is the first report showing that an endogenous G_i-coupled GPCR successfully couples to a chimeric G protein and thereby can be linked to the FLIPR readout. Future studies using other endogenous G_i-coupled receptors may provide valuable alternatives to the standard recombinant receptor expression technologies.

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