Chapter 7

Compound screen on orphan G protein-coupled receptor GPR88

The work described in this chapter was done at the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France, under supervision of Prof. Dr. B. L. Kieffer and Dr. J.A. Becker; and at AstraZeneca R&D, Montréal, Québec, under supervision of Dr. E. Grazzini and Dr. P. LaPlante.

Abstract

GPR88 is a G protein-coupled receptor without known endogenous or synthetic ligands. However, multiple genetic and expression studies suggest that GPR88 may be implicated in psychiatric disorders such as schizophrenia, depression and bipolar disorder. We performed a ligand identification screen testing over 4000 small molecules and peptides. This was done in a 384-well format, using changes in intracellular calcium levels detected with a Fluorescent Imaging Plate Reader (FLIPR) as the readout. Since the G protein coupling specificity of GPR88 is unknown, HEK293s cell lines stably co-expressing the receptor with $G\alpha_{16}$ or $G\alpha_{qi5}$ were used, as well as a native HEK-GPR88 cell line. In the calcium screen 46 hits were identified, with responses occurring in different GPR88-expressing cell lines giving no clue as to the G protein coupling specificity. In validation assays, dose-response curves could be obtained for papaverine, an opium alkaloid. However, this compound also provoked calcium responses in HEK cells that were not transfected with GPR88. Thus, identification of a ligand, either endogenous or synthetic, for GPR88 remains an unmet challenge.

Introduction

Since approximately 30% of all drugs currently on the market target rhodopsin-like G protein-coupled receptors (GPCRs), this protein family is of great interest for drug research. However, from a total of 284 human non-olfactory class A receptors, only about 50 are currently targets of marketed drugs. Even more strikingly, 63 class A GPCRs have no known endogenous ligand or function (see also Chapter 1). Unlocking the full therapeutic potential of all these receptors could bring highly significant advances to the treatment of a variety of diseases. A first step towards this goal can be the identification of (endogenous) ligands for orphan receptors.

This study focused on the orphan receptor GPR88, which was initially identified as a striatum-specific receptor [1-2]. We have also reported it as a gene with enriched expression in the central extended amygdala [3]. GPR88 is of special interest, since it may be implicated in several psychiatric and neurological disorders. Mice lacking this receptor, which is predominantly expressed in the brain, show a schizophrenia-like phenotype with impaired prepulse inhibition of startle (PPI), increased apomorphine-induced climbing and stereotypy, and increased amphetamine-stimulated locomotor hyperactivity [4]. These phenotypes were sensitive to treatment with antipsychotics. Lack of GPR88 modulated the striatal dopaminergic system by increasing the functional sensitivity of dopamine D_2 receptors and increasing DARPP-32 Thr-34 phosphorylation. Other hints regarding the role of GPR88 come from gene expression studies. We and others have reported regulation of the rodent GPR88 gene in several brain regions after treatment regimens with addictive drugs, antidepressants and mood regulators [5-10]. For example, a 13-fold upregulation was seen in nucleus accumbens dopaminergic neurons after chronic treatment of mice with the tricyclic antidepressant amitriptyline [6]. Other antidepressant treatments, including fluoxetine, sleep deprivation and electro-convulsive therapy, were shown to cause changes in GPR88 expression in the hypothalamus [8]. In our laboratory, GPR88 upregulation was seen in mouse central extended amygdala after chronic morphine treatment [5]. Changes in GPR88 expression levels were also observed in lactating rats [11], in rodent models of depression [12] and Parkinson's disease [13], and in human Huntington's disease brains [14].

GPR88 is most closely related to the biogenic amine receptors, especially the β_1 and β_3 adrenergic receptors, and the 5-HT1D receptor. However, even to these receptors the sequence homology is relatively low (up to 27% amino acid identity) and it lacks the aspartic acid in the third transmembrane (TM) domain that is the hallmark of biogenic amine receptors. A phylogenetic analysis using only 30 residues predicted to line the TM binding cavity of GPCRs detected similarities between the class A receptor GPR88 and the class C receptors $GABA_B$, taste and metabotropic glutamate receptors [15]. In this analysis GPR88 was the only receptor clustering to a different receptor class. Possibly, GPR88 and certain class C receptors bind structurally related ligands in their TM binding pockets (which is the allosteric pocket for class C receptors).

In our ligand identification campaign, we screened over 4000 compounds, including naturally occurring and synthetic small molecules and peptides, for activation of human

GPR88. Elevation of intracellular calcium concentrations was the functional readout in our screen. The G protein coupling specificity of GPR88 is unknown, so to obtain calcium responses we co-expressed the receptor with the chimeric G protein $G\alpha_{qi5}$ or the promiscuous $G\alpha_q$-family protein $G\alpha_{16}$, thus forcing GPR88 to couple to the G_q pathway, regardless of the native coupling preference [16-18].

Materials and Methods

Generation and characterization of HEK293s-hGPR88 cell lines

Construction of a hGPR88 - N-Flag - signal-sequence plasmid

Human GPR88 cDNA cloned in pcDNA 3.1+ (Neomycin) was bought from the cDNA Resource Center from the University of Missouri-Rolla. A modified influenza hemaglutinin signal sequence [19] and a Flag tag were inserted upstream of the receptor sequence. The ~240 N-terminal bases of the GPR88 gene were amplified by PCR using a Signal sequence-Flag tag sense primer and Expand High Fidelity polymerase (Roche). The sequences of the primers were: Sense: GCGAATTCCACCATGAAGACGATCATCGCCCTGAGCTACATCTTCTGC CTGGTATTCGCCGACTACAAGGACGATGATGACGCCACCAACTCCTCCTCCACATCCACC; Reverse: GGACGACACGAGATAGATGACCATGCCGTT. In total 50 ng of matrix was used with 100 pmol of each primer in a total volume of 100 µl. An initial denaturation step of 3 min at 94 °C was followed by 20 amplification cycles (30s 94 °C; 30s 55 °C; 20s 72 °C). A phenol-chloroform precipitation was carried out after which the product was taken up in 10 mM Tris pH 7.4 / 1 mM EDTA. Then, 2 µg was digested with 20 IU of EcoRI and AgeI and the resulting product was inserted into the pcDNA3.1+ plasmid bearing the cDNA of GPR88 that was pre-digested with the same enzymes. The ligation was carried out with 50 ng linearized plasmid and 2 ng fragment in 10 µl of commercial buffer with 3 IU ligase (Biolabs) for 4h at 4 °C. The product was used to transform competent Escherichia Coli XLM Blue cells and the plasmid was isolated from an ampicillin-resistant colony. Sequencing confirmed that the plasmid contained the tagged human GPR88 sequence.

Generation of stable cell lines expressing hGPR88- Flag-signal sequence

Transfection of HEK293s cells

The day before transfection 2.5-4.10^6 human embryonic kidney (HEK) 293s cells were seeded in a 10 cm diameter cell culture plate. For transfection, 16 µl JetPEI transfection reagent (Polyplus transfection) in 500 µl NaCl 150 mM was added to a solution of 8 µg pcDNA3+/GPR88 in 500 µl NaCl 150 mM, and incubated for 15-30 min at RT. The empty vector pcDNA3 was used as a negative control. Fresh cell culture medium was added to the cells and 1 ml of the transfection mixture was added to each plate. To obtain a stable cell line, Geneticin 418 was added to the medium on day 4.

Cell lines and cell culture conditions

Three HEK293s cell lines from AstraZeneca R&D Montréal were used: HEK293s nontransfected cells, HEK293s stably expressing chimeric $G\alpha_{qi5}$ and HEK293s stably expressing $G\alpha_{16}$. Plasmids used for construction of these cell lines were pCEP-$G\alpha_{qi5}$-HA from Molecular Devices and pcDNA3.1-$G\alpha_{16}$ from AstraZeneca Montréal.

Cells were grown at 37 °C and 5% CO_2, in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1000 units/ml penicillin G and 1 mg/ml streptomycin. The HEK-Gα_{qi5} cells were grown in the presence of 300 μg/ml Hygromycin B and HEK-Gα_{16} cells were grown in the presence of 350 μg/ml Zeocin. Geneticin 418 sulfate was used at a concentration of 500 μg/ml for selection of the cells stably expressing GPR88.

Enrichment of hGPR88-Flag expressing cells by fluorescent automated cell sorting (FACS)

Cells were detached using 10 mM EDTA and washed two times in PBS, followed by 30 min incubation on ice with the monoclonal antibody anti-Flag M2 (Sigma Aldrich) (1:20000 in PBS 1% BSA, filter sterilized). Cells were washed two times with PBS 1% BSA and incubated 30 min on ice with the secondary antibody goat F(ab')2 fragment antimouse IgG (H+L)-FITC (Beckman Coulter) (1:200 in PBS 1% BSA, filter sterilized). After two final washes in PBS the cells were diluted at 5.10^5 cells/ml in culture medium. Cell clumps were removed with a sterile 50 μm Filcon filter (Consul T.S.). Cells were sorted on a Becton Dickinson FACS DiVa using a 100 μm gauge. FITC-positive cells with fluorescence ≥ 1000 units were deflected into tubes containing 0.5 ml culture medium.

Assessment of GPR88-Flag expression in HEK293s cell lines

Flow cytometric analysis

Preparation of the cells for flow cytometric analysis was performed as described for FACS, but not under sterile conditions. The amount of fixed FITC was analyzed on a Becton Dickinson FACS DiVa as described, or on a Becton Dickinson FACSCalibur using essentially the same settings. A total of 3.10^4 single cells were included in each analysis.

Immunocytochemistry

Cells were seeded in 6-well plates containing glass coverslips coated in poly-L-ornithine or poly-D-lysine at 2.10^5 cells per well. After 1-2 days cells were washed three times in PBS and fixed for 15-60 min with 4% PFA in PBS at room temperature. Cells were washed three times with PBS and incubated overnight at 4 °C with the monoclonal anti-Flag M2 antibody (Sigma Aldrich) (1:20000 in PBS 1% BSA 0.1% Tween20). Cells were washed three times with PBS 0.1% Tween20. The glass slides were covered with the secondary antibody goat F(ab')2 fragment antimouse IgG (H+L)-FITC (Beckman Coulter) (1:200 in PBS 1% BSA 0.1% Tween20). Alternatively, the secondary antibody goat anti mouse Alexa fluor 488 (1:800 in PBS 1% BSA 0.1% Tween20) was used. After two washes in PBS the cells were DAPI stained (1:10000 in PBS 5 min at RT). After final washing (3xPBS, 1xH_2O) the glass slides were air dried and mounted on microscope slides using Mowiol. Alternatively the wet slides were mounted with Aqua Poly/Mount (Polysciences) and sealed with nail polish.

Compound library screening for activation of GPR88

Calcium mobilization experiments using Fluorescent Imaging Plate Reader (FLIPR)

Cells were detached with Accutase (Innovative Cell Technologies) and resuspended in 10 ml complete medium without selection agents. Cells were seeded in 384-well black, clear-bottom poly-D-lysine coated plates (Becton Dickinson BioCoat) at approximately 10^4 cells/well in 50 µl, titrated to obtain a confluence of 80% the following day. Then, Fluo-4 AM (Invitrogen) (8 µM) and Pluronic F-127 (Molecular Probes) (0.08%), both from DMSO stocks, were dissolved in the assay buffer (HBSS containing calcium and magnesium and 5 mM glucose (Wisent) with 0.1% BSA and 20 mM Hepes added fresh, pH 7.4). The final concentration of DMSO in the loading solution was 0.8%. Before loading, part of the medium was removed with an Embla 384 cell washer (Molecular Devices) so that 25 µl residual medium was left in each well. Then, 25 µl loading buffer was added to each well using a Multidrop 384 (Thermo Scientific). The plates were incubated 1 h at 37 °C with 5% CO_2. After the incubation period the cells were washed four times in assay buffer using the Embla cell washer, leaving 25 µl buffer per well after the wash protocol. Then the cells were allowed to rest at least 5 min at room temperature in the dark. The plates were read in a FLIPR^{384} system (Molecular Devices) using the following settings: pipettor height: 30 µl; dispense speed: 15 µl/s; fluid volume: 12.5 µl; exposure length: 0.4 s; filter #1 (510-570 nm). The baseline was read for 10 seconds, and then 12.5 µl of the ligands (final concentration 1 µM) was automatically added to the cell plate. The DMSO concentration at the cells never exceeded 2%. The total reading time was 3 min. The data was analysed using the SoftMax Pro software (Molecular Devices).

Calcium mobilization experiments using Flexstation 3

The experiments were carried out essentially as for FLIPR with the following exceptions. Cells were plated in 96-well black, clear-bottom poly-D-lysine coated plates (Becton Dickinson BioCoat) at about 5.10^4 cells/well in 200 µl. The assay buffer composition was HBSS (Sigma) with 0.1% BSA and 20 mM Hepes added fresh. The loading solution contained 4 µM Fluo-4 AM (Invitrogen) and 0.04% Pluronic F-127 (Molecular Probes) in a mixture of 50% assay buffer and 50% of the usual culture medium without selection antibiotics. Before loading, the medium was removed by decanting. Then, 100 µL loading buffer was added to each well using a multichannel pipette. After the incubation period the cells were washed three times in assay buffer (200 µl/well), and then 40 µl assay buffer was added per well. The plates were analyzed in a Flexstation 3 system (Molecular Devices). Fluo-4 was excited at 488 nm and the emission at 520 nm was detected. The 'Flex' reading mode was used and the detection sensitivity was set to 'high'. The baseline was read during 25 s and then 20 µl of the ligands was automatically added to the selected wells of the cell plate (pipetting speed 1; pipette height 50 µl). For compounds cherry-picked from the compound plates this resulted in a final compound concentration of 1 µM.

Compound collection

The compounds tested in the ligand identification campaign were stored at 3 μM in aqueous solution at -80 °C, in 96-well compound plates. Prior to the experiments four 96 well plates were combined into one 384 well plate using a Biomek FX robot (Beckman Coulter). The compound libraries tested were the Sigma-RBI LOPAC$^{640\ TM}$ ligand collection (640 compounds), the Prestwick Chemical Library (880 compounds), a library of endogenous and predicted peptides (1611 compounds) and the Jerini (JPT) RF-amide collection (1000 compounds), yielding a total of 4131 compounds.

Results

Generation and characterization of HEK293s-hGPR88 cell lines

Construction of a hGPR88 - N-Flag - signal-sequence plasmid

In order to facilitate immunostaining and membrane expression of GPR88 in HEK293s cell lines we inserted a Flag-tag and a modified influenza hemaglutinin signal sequence [19] upstream of the receptor sequence. The full GPR88 sequence was resistant to PCR amplification, so a cloning strategy was developed where only the 240 N-terminal bases of the GPR88 genes were amplified by PCR to insert the Flag-tag and signal sequence. Cloning of the product into pcDNA3.1+ bearing the cDNA of GPR88 yielded the correct plasmid as verified by digestion and sequencing.

Subcellular localization of transiently expressed GPR88

We verified by immunocytochemistry that hGPR88 with Flag tag and signal sequence was expressed on the plasma membrane in transiently transfected HEK293s cells. We used transient transfection with a Flag-tagged human delta opioid receptor (DOR-Flag) as a positive control. Cells transfected with GPR88-Signal-Flag and DOR-Flag showed both intracellular and membrane staining in about 50% of the cells 48 h after transfection (data not shown).

Generation of stable cell lines expressing hGPR88- Flag-signal sequence

Since the G protein coupling specificity of GPR88 is unknown, co-transfection with promiscuous $G\alpha_{16}$ or with chimeric $G\alpha_{qi5}$ was employed to facilitate coupling to the

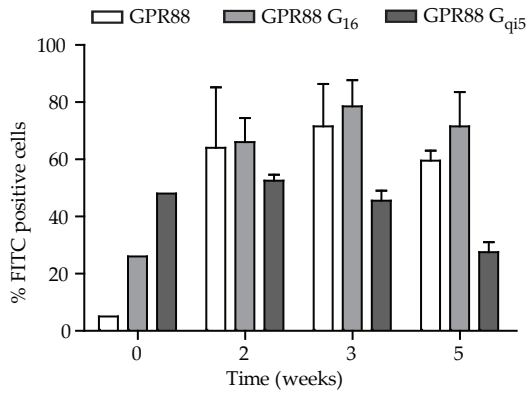

Figure 1. Expression of GPR88-Flag in the three stable cell lines estimated by flow cytometric analysis of 30000 cells (N=2). Timepoint 0 represents the values before FACS sorting. All cells with fluorescence above 1000 units were considered FITC-positive.

calcium pathway. Native HEK293s cells and HEK293s cell lines stably expressing either $G\alpha_{16}$ or $G\alpha_{qi5}$ were transfected with the pcDNA3+/hGPR88/Flag/signal sequence plasmid. Control cell lines were generated by transfection with empty pcDNA3 vector. Four days after transfection the appropriate selection agents were added. Per cell line, twelve resistant colonies were isolated and stored, whereas all other resistant colonies (>100 per cell line) were pooled. Six weeks after transfection the expression level of GPR88-Flag in the resistant cell pools was determined by labeling with a primary anti-Flag antibody and a secondary antibody labeled with FITC, followed by flow cytometry. At the first analysis the transfected HEK293s and HEK293s $G\alpha_{qi5}$ cell lines had approximately 5% FITC-positive cells, whereas the transfected HEK293s $G\alpha_{16}$ cell line had 25% FITC-positive cells.

Enrichment of GPR88-Flag expressing cells by fluorescent automated cell sorting

In order to obtain higher GPR88 expression, FITC positive cells were selected by fluorescent automated cell sorting (FACS). HEK cells transfected with the empty vector showed fluorescence below 800 units, so cells with fluorescence above 1000 units were considered FITC-positive. The negative control cell lines HEK293s, HEK293s-$G\alpha_{16}$ and HEK293s-$G\alpha_{qi5}$

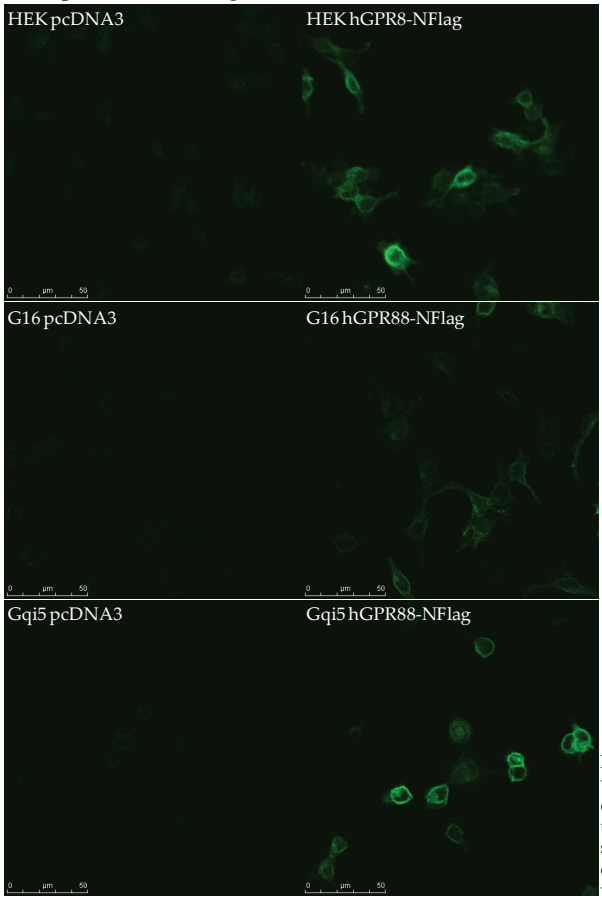

Figure 2. Immunocytochemical staining with an anti-Flag antibody in order to demonstrate expression of GPR88-Flag at the cell membrane of HEK293 cells. The same staining was performed on control cell lines transfected with empty pcDNA3 vector (left panels).

transfected with pcDNA3 were also passed through FACS as a mock-sorting.

The enriched HEK-Gα_{qi5}-GPR88 cells lost expression of GPR88 in the first week after sorting. We therefore analyzed the 12 clones isolated after the Geneticin selection, of which two had a moderate percentage of GPR88 expressing cells (14 and 17% FITC positives) and one a low percentage (0.5%). The three positive colonies were pooled and the positive cells were isolated by FACS sorting.

The stability of the GPR88-Flag expression over time was monitored by flow cytometric analysis (figure 1). The expression of GPR88 was fairly stable, although the percentage of expressing cells diminished in the Gα_{qi5} GPR88 cell lines.

Monitoring of GPR88-Flag expression by immunocytochemistry

The expression of GPR88-Flag over time was also assessed by immunocytochemistry using a primary anti-Flag antibody and a fluorescent secondary antibody. Three weeks after FACS enrichment, the percentage of positive cells was estimated at 45%, 50% and 40% for the HEK cells containing GPR88 alone, GPR88-G_{16}, and GPR88-G_{qi5}, respectively. Prior to the start of experiments on a new batch of cells the expression level of GPR88-Flag was checked by immunocytochemistry, an example of which is shown in figure 2. The expression levels were checked again at the end of each series of experiments. Typically, membrane staining was observed in approximately 50% of the cells stably transfected with GPR88-Flag. In some cases, especially in the cells transfected with Gα_{qi5}, a strong signal was observed around the nucleus.

Compound library screening for activation of GPR88

We performed a ligand identification campaign for GPR88. The compounds we tested in the screen were stored in ready-for-use compound plates at -80°C. In total, 4131 compounds were tested at least in duplicate at a final concentration of 1 μM. The compound libraries we tested were the Sigma-RBI LOPAC$^{640\ TM}$ ligand collection, the Prestwick Chemical Library, an in-house library of endogenous and predicted peptides and the Jerini (JPT) RF-amide collection.

Calcium mobilization primary screen using FLIPR

The collection of 4131 compounds was tested for the capability of provoking a calcium response through activation of GPR88 in native HEK cells or cells co-expressing Gα_{16} or Gα_{qi5}. Corresponding cell lines transfected with empty vector pcDNA3 were used as negative controls. The experiments were conducted on attached cells in 384 well plates, using a FLIPR system (Molecular Devices) for detection of the fluorescent signal of the calcium indicator Fluo-4, and for robotic addition of the test compounds. The DMSO concentration never exceeded 2% and controls showed that this amount did not change the fluorescence signal. The fluorescence maxima and the shape of the curves were examined by eye to conclude whether GPCR activation took place. A maximum fluorescence below 1000 fluorescence counts was considered background. On each compound plate positive and negative (buffer only) controls were included. As positive controls, the agonists endothelin-1, vasoactive intestinal protein (VIP) and pituitary adenylate cyclase-

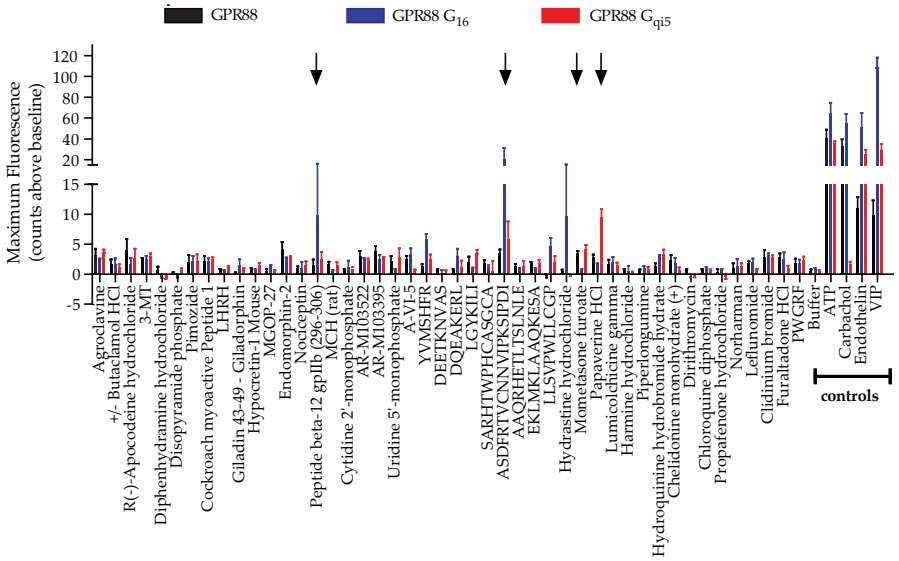

Figure 3. Maximum fluorescence responses in a calcium mobilization assay for 46 hits from the primary screen. The compound solutions were cherry-picked from the screening plates (final concentration 1 μM). Average values (± SEM) (N≥2) are shown. The arrows indicate compounds yielding a robust response in this assay.

activating peptide (PACAP), capable of activating endogenous HEK293s GPCRs, were used. All compounds were tested in two independent single-point experiments at a final concentration of 1 μM.

The calcium responses obtained from potential hits were low to moderate. Out of 71 calcium responses to the most promising compounds only 5 responses were above 3000 fluorescence counts, and 47 reached their maximum below 2000 fluorescence counts. Some compounds provoked calcium responses in the cell line HEK293s-Gα_{qi5}-GPR88, some in the cell line HEK293s-Gα_{16}-GPR88 and yet others in the cell line HEK293s-GPR88, whereas several compounds stimulated more than one cell line. Only the hits peptide beta-12,

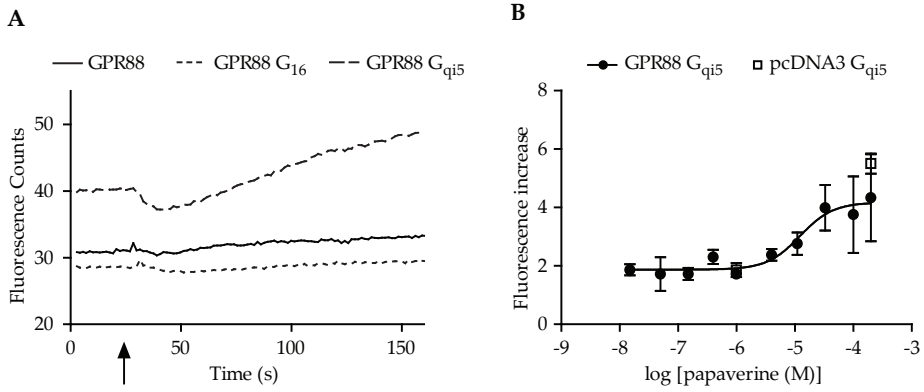

Figure 4. A. Calcium mobilization responses to papaverine in HEK cells expressing GPR88-Flag. This experiment was performed with compound solution cherry-picked from the screening plates (final concentration 1 μM). The arrow indicates the time of compound addition. **B.** Dose-response curves of papaverine from calcium mobilization assays. Mean data from two independent experiments is shown. The compounds were also tested on nontransfected cell lines at 1 and 200 μM.

chelidonine monohydrate (+), chloroquine diphosphate, clidinium bromide and PWGRF could be confirmed in two independent experiments. When the average responses over the two experiments were taken into account, 26 compounds qualified as hits. Several other compounds that provoked a robust calcium response, but only in one of the two experiments were also selected for further study, bringing the total to 46 hits.

Hit validation assays

The compounds that were selected from the screen performed on FLIPR were entered in a secondary screening round that was performed in a similar manner but using a Flexstation 3 (Molecular Devices). First, the ideal conditions for the calcium experiments at the Flexstation 3 were determined. In order to keep the compound use as low as possible a working volume of 60 μl was selected instead of the recommended 150 μl, which did not affect the reproducibility and the signal-to-noise ratio.

In total, 46 promising compounds were tested twice at the Flexstation 3 on the three cell lines expressing GPR88. Figure 3 shows the maximum responses that were observed in these experiments. Most compounds did not provoke a calcium response. Small responses were seen in one experiment for papaverine (Figure 4A) and mometasone furoate and moderate responses were seen for peptide beta-12 and the peptide ASDFRTVCNNVIPKSIPDI. The responses to peptide beta-12 administration could not be reproduced in two further experiments using this compound from the same and a different compound plate. Fresh solutions were prepared for testing mometasone furoate, papaverine hydrochloride and the peptide ASDFRTVCNNVIPKSIPDI at higher concentrations. Mometasone furoate and papaverine were tested at ten concentrations ranging between 15 nM and 200 μM, and the peptide was tested at 1 and 10 μM (N=2). No responses were seen to either concentration of ASDFRTVCNNVIPKSIPDI. For mometasone furoate small responses were observed in all GPR88 expressing cell lines at the highest compound concentration (200 μM), but the responses were also observed in the HEK293s pcDNA3 cells and no dose-response curve could be obtained. For papaverine, a dose-response curve was observed in the cell lines containing $G\alpha_{qi5}$ (figure 4B) and $G\alpha_{16}$ (not shown), but the small responses observed were similar in the pcDNA3-transfected cells. Therefore we must conclude that no GPR88-mediated responses could be demonstrated.

$GABA_B$ allosteric modulators as putative GPR88 ligands

As described in the introduction, phylogenetic analysis suggests that ligands for GPR88 might bear a certain resemblance to allosteric modulators of the $GABA_B$ receptor. Therefore, we tested three commercially available compounds that are known to modulate $GABA_B$ (CGP7930, CGP13501 and GS39783) on the GPR88 expressing cell lines, using the Flexstation 3. The compounds did not provoke a calcium response, even at a concentration of 10 μM (data not shown).

Discussion

Despite a decade of academic and private research efforts, GPR88 remains an orphan receptor with no known synthetic or endogenous ligands. The only hint at compounds

activating GPR88 was found in the patent literature, where ligands for GPR84 were described with limited selectivity towards this receptor over GPR88 [20]. The ligand identification screen we performed yielded 46 hits out of 4131 compounds tested, but none of the hits could be confirmed as a GPR88 agonist.

Our failure to identify an agonist for GPR88 may be due to the limited number of compounds we tested. Compound collections of tens to hundreds of thousands in size are no exception [21], and Johnson and coworkers even screened over a million molecules [22]. Major GPCR ligand classes were represented in our compound collection but GPR88 may display specific binding to a ligand or ligand class that was not included. It is possible that this receptor, which does not show high sequence similarity to any GPCR subfamily [23-25], binds to an unusual or unknown signaling molecule. Testing at a higher concentration could also increase the chances of identifying a ligand. Even a low potency agonist could give valuable clues regarding downstream signaling, and at the same time serve as a starting point in the search for a more potent ligand. Furthermore, some GPCRs may not bind with a high affinity to their endogenous ligands, as seems to be the case for the recently deorphanized hydroxy-carboxylic acid receptor family [26]. Despite the added cost, screening at a higher concentration may be worth it.

A major difficulty in deorphanization screens is the inherent absence of real positive control conditions to test the detection of receptor activation by an agonist. Accessory proteins potentially required for GPR88 function could be absent in HEK293s cells. Although we confirmed that the receptor was expressed at the membrane and that the signals from endogenous GPCRs on HEK cells were detected correctly, it is also conceivable that our screening methods were not ideal for the detection of GPR88 activation. We cast our net wider by performing a second screen, where the read-out was a membrane depolarization instead of a change in intracellular calcium concentration. This screen, which is not further described here, yielded 77 hits of which none could be confirmed in validation experiments.

GPR88 so far resisted attempts at deorphanization, not only by us but by several other groups as well, judging from personal communications and the website of the Michael J. Fox Foundation, which awarded a grant for exploring GPR88 as a target for the treatment of Parkinson's disease in 2008, which did not yield any ligands (http://www.michaeljfox.org/research_MJFFfundingPortfolio_searchableAwardedGrants_3.cfm?ID=432). It could be that GPR88 is one of the 'real' orphans that do not have an endogenous ligand, nor need one to function. The physiological relevance of such a GPCR could be as a chaperone for another receptor, similar to $GABA_{B2}$ [27] or as a constitutively signaling protein, like GPR3 (GPR12), a G_s-coupled orphan receptor with an apparently ligand-independent role in the female reproductive system [28-29].

Another example of an orphan receptor functioning independently from a ligand is GPR50 dimerizing with the MT_1 and MT_2 melatonin receptors, which prevents melatonin signaling in the case of MT_1 [30]. In fact, unpublished data from R. Massart, P. Sokoloff et al. (briefly summarized in this editorial: http://scielo.isciii.es/pdf/ejpen/v24n1/editorial.pdf) suggest an unusual and possibly ligand-independent function for GPR88: the receptor may have a role as an intranuclear, chromatin-associated protein modulating

developmental nuclear transportation in differentiating cortical neurons. However, the same group also reported localization of GPR88 outside of the nucleus in mature striatal medium spiny neurons, preferentially in post-synaptic domains [13].

A first step towards future deorphanization of GPR88 could be the elucidation of its downstream signaling. Receptor overexpression or mutagenesis could be used to trigger enhanced basal or constitutive signaling. Further screening could then be limited to one GPR88-expressing cell line and one assay only, while we screened on three cell lines in two assays. Screening in a striatal, neuronal cell line [see for example 31, 32] expressing GPR88 naturally would further increase the chances of success, since all necessary machinery for receptor signaling is presumably expressed in these cells.

In GPCR deorphanization, the identification of the first ligand is an enormous hurdle. When this is overcome, further ligands can be identified with relative ease, and the role of the receptor in (patho-)physiology can be explored in more detail. In my opinion, new ligands for orphan GPCRs, even if they have a low potency, should be made public as soon as possible after discovery. This would be in the interest of the scientific community as a whole, and the pharmaceutical industry in particular.

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