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A FLIPR Assay for Evaluating Agonists and Antagonists of GPCR Heterodimers

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Abstract

Calcium signaling plays a major role in the function of cells. Measurement of intracellular calcium mobilization is a robust assay that can be performed in a high-throughput manner to study the effect of compounds on potential drug targets. Pharmaceutical companies frequently use calcium signaling assays to screen compound libraries on G-protein-coupled receptors (GPCRs). In this chapter we describe the application of FLIPR technology to the evaluation of GPCR-induced calcium mobilization. We also include the implications of GPCR hetero-oligomerization and the identification of heteromeric receptors as novel drug targets on high-throughput calcium screening.

Keywords

Calcium mobilization; G-protein-coupled receptor; GPCR; High-throughput screening; HTS; Heteromer; Oligomerization; Bivalent ligands

1 Introduction

The high-throughput screening (HTS) of large libraries of drug-like compounds is a key aspect of drug discovery. G-protein-coupled receptors (GPCRs) form one of the largest families of drug targets to which HTS is regularly applied (1). GPCRs predominantly exert their function by coupling to heterotrimeric G proteins. Depending on the G_α subunit ($G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha 12}$), the receptor will signal via adenylyl cyclase/cAMP, phospholipase C/ Ca^{2+} , or Rho (2). The activity of receptors that signal through $G_{\alpha q}$ to release Ca^{2+} can be easily monitored in a HTS environment using the fluorescence-based FLIPR calcium assay (Molecular Devices, Sunnyvale, CA). The existence of promiscuous G proteins ($G_{\alpha 16}$) and chimeric G proteins ($G_{\alpha qs}$, $G_{\alpha qi}$) that can provide calcium readouts for GPCRs that would otherwise not signal via the $G_{\alpha q}$ pathway (Fig. 1) adds to the versatility of the FLIPR assay (3, 4).

Traditionally, GPCRs were believed to function as monomers (a single receptor binding one G protein), but, over the past decade, a new consensus has emerged in light of growing evidence that GPCRs can form oligomeric structures. GPCR oligomers can result from the association of one type of receptor with itself (homomers) or from interactions among different receptors (heteromers) (5). Current data suggests that receptor heteromers can have unique pharmacological properties (6, 7) and may be viable drug targets. Thus far, several ligands have been designed or discovered that can bind heteromers. These ligands fall into two groups: monovalent and bivalent (two monophores connected by a linker of appropriate length) ligands. For example, 6'-GNTI is a monovalent ligand that shows potent activity

only at the delta-kappa-opioid receptor heteromer (8), whereas the MDAN series are bivalent ligands consisting of a mu-opioid receptor agonist and a delta-opioid receptor antagonist linked by spacers of different lengths (9), some of which produce a lower degree of dependence than morphine or other monovalent ligands that act on mu-opioid receptors alone (Fig. 2).

This chapter describes the application of FLIPR to both conventional as well as bivalent ligands.

2 Materials

2.1 Cell Culture

1. Human embryonic kidney cells (HEK-293) (ATCC, Manassas, VA).
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, Carlsbad, CA).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA).
4. Phosphate buffered saline solution (PBS).
5. Probenecid.
6. Sodium hydroxide.
7. 10× Hank's balanced salt solution (HBSS).
8. 1 M HEPES, pH 7.2–7.5.
9. Poly-D-Lysine.
10. Clear bottom 96-well black plates (Costar, Corning, NY).
11. FLIPR calcium assay kit (Molecular Devices).
12. FlexStation pipette tips (96, clear) (Molecular Devices).

2.2 Transfection of HEK-293 Cells with GPCR and Chimeric G Proteins

1. 1 µg/µl plasmid DNA carrying GPCR of choice (e.g., pcDNA3.1-DOR).
2. 1 µg/µl Gqi5-myr (see Note 1).
3. Lipofectamine (Invitrogen).
4. Opti-MEM (GIBCO, Invitrogen).

3 Methods

3.1 Growing and Seeding Cells

1. HEK-293 expressing the receptor(s) of choice are grown to at least 80% confluent in 10 ml DMEM/10% FBS in a 75 cm² cell culture flask in an incubator at 37°C/5% CO₂.

2. Count the cells before seeding into a 96-well clear bottom black plate (see Note 2).
3. Dilute cells to 700,000 cells/ml in DMEM/10% FBS and resuspend by gently pipetting up and down (see Note 3).
4. Transfer 100 μ l of the diluted cell suspension to each well in the 96-well plate (see Note 4).
5. Incubate the cells for one day in the CO₂-incubator at 37°C/5% CO₂.

3.2 Transfection of Cells with GPCR and/or Chimeric G Protein (Optional, See Note 5)

1. Mix 100 ng of DNA with 50 μ l (2 mg/ml) Opti-MEM per well.
2. Mix 0.5 μ l Lipofectamine with 50 μ l (10 μ g/ml) Opti-MEM per well and incubate for 5 min at room temperature (see Note 6).
3. Add the DNA solution to the Lipofectamine solution and incubate for 20 min at room temperature.
4. Aspirate the medium from the cells in the 96-well plate (see Note 7).
5. Gently add 100 μ l Opti-MEM to each well using a multichannel pipette.
6. Add 100 μ l of the DNA/Lipofectamine solution to each well (final volume is 200 μ l) and incubate for 4–5 h in the CO₂-incubator at 37°C/5% CO₂.
7. Aspirate the DNA/Lipofectamine solution, replace it with 100 μ l DMEM/10%FBS, and incubate for one day in the CO₂-incubator at 37°C/5% CO₂ (see Note 8).

3.3 Calcium Mobilization Measurement Using the FlexStation

1. Add 10 ml assay buffer to one vial of FLIPR reagent per 96-well plate and mix by vortexing. (see Note 9).
2. Add 100 μ l/well FLIPR reagent to the cells in the 96-well plate and incubate for 1 h in a CO₂-incubator at 37°C /5% CO₂ (see Note 10).
3. Prepare the ligands (see Subheading 3.4 below).
4. Optionally, add antagonist to the cells at the appropriate time before measurement (see Note 11).
5. Turn on the FlexStation about 15 min prior to measurement to set up the assay protocol and allow the FlexStation to reach the proper temperature. Select the correct compound plate type, assay plate type, which wells to measure, and the speed and depth at which agonist will be transferred (see Note 12).
6. Set up the protocol to add agonist 10–20 s after the start of the measurement and measure for 2 min with 1.5 s intervals (79 data points). Wavelengths are set at 482 nm for excitation and 525 for emission.

7. Place ligands, cells, and pipette tips in the FlexStation and start measurement (see Note 13).

3.4 Ligand Preparation

3.4.1 Agonist

1. The ligand plate can be prepared after the calcium dye has been added to the cells, during the 60 min waiting period (see Subheading 3.3).
2. Each concentration in the ligand dilution series needs to be prepared at a 5× concentration to account for the dilution that occurs upon mixing with the cells. The dilution series is prepared in the calcium assay buffer (see Note 14).
3. The dilution series is prepared in triplicate on a 96-well plate. The top row can be reserved for solvent controls (for example, buffer only or 0.1% DMSO, see Note 15). Seven concentrations of four different ligands can then be accommodated (alternatively, eight concentrations of three of the ligands can be used if the entire top row is not needed for controls, but three wells are always reserved for the buffer only control).
4. Prepare a solution of the highest concentration of ligand. For a standard log step dilution series pipette, for example, 200 µl of solution into well A1. Fill wells A2–A8 with 180 µl of assay buffer (see Note 16). To prepare the dilution series take 20 µl out of A1 and transfer to A2, mix well, and take 20 µl from A2 and transfer to A3 and so forth. To include 0.5 log steps, prepare a 5× and a 1.67× concentration for the highest concentration of ligand. Transfer this solution to the 5× stock in well A1 and the 1.67× in well A2. Prepare tenfold dilutions A1 to A3 to A5, A2 to A4 to A6, etc.
5. After all ligands have been prepared, and just prior to insertion in the FlexStation, briefly place the plate on a shaker to eliminate air bubbles and ensure mixing.

3.4.2 Antagonist

1. Calcium mobilization does not provide a direct measure of antagonism or inverse agonism. However, antagonism can be determined indirectly by measuring inhibition of agonist-induced calcium release. The antagonist can be added either prior to measurement (10–15 min beforehand), or a solution containing both the agonist and antagonist can be prepared for simultaneous addition (see Note 17) (Fig. 3).

When multiple dose–response curves are produced in the presence of an increasing concentration of antagonist, the potency of the antagonist can be measured in the form of a pA_2 value obtained from a Schild plot.

3.4.3 Bivalent Ligands

1. Both biochemical (e.g., cross-linking and immunoprecipitation studies) and resonance energy transfer (e.g., bioluminescence resonance energy transfer,

fluorescence resonance energy transfer) techniques have shown that receptor oligomerization occurs for many receptors in heterologous expression systems. Additionally, current research implicates receptor heteromers as potential drug targets, and thus a HTS assay to detect heteromer-selective ligands will be needed. To screen for heteromer-selective ligands, the ligand needs to be tested on three different cell lines: cells expressing receptor 1, cells expressing receptor 2, and cells co-expressing receptor 1 and 2. Using the calcium assay we have been able to identify 6'-GNTI as a potent kappa-delta-opioid heteromer-selective agonist (8). The potency (and thus, the functional activity) of this ligand in cells co-expressing KOR and DOR is distinct from that in cells expressing only KOR or DOR. 6'-GNTI is a weak partial agonist at kappa-opioid receptors (and, thus, functional antagonist in the presence of full agonists such as U50,488), and a potent antagonist at DORs (Fig. 4). On the other hand, we predict MDAN-19 (9), a bivalent ligand comprised of a MOR agonist and a DOR antagonist, to be a potent agonist in cells expressing MOR and a potent antagonist in cells expressing DOR. However, in cells expressing MOR and DOR, it could be either an agonist, or antagonist, or partial agonist, depending on which receptor response predominates in the context of a heteromer.

2. The G protein to which a heteromer couples may be different than the G protein associated with the monomeric receptor(s). Initially, it may not be possible to predict through which G protein the heteromer signals. Thus, the assay can be performed both with and without transfection of a known chimeric G protein. This assay can therefore be used to determine whether heteromerization alters specificity of G protein coupling. For example, neither dopamine D1 nor dopamine D2 receptors couple to G_q when expressed alone (they are G_{s/olf} and G_{i/o} coupled receptors, respectively). However, when these two receptors are co-expressed (and presumably generate heteromers), a dopamine-induced calcium signal is produced, implying that this heteromer couples to G_q. In addition, it is possible that various ligands at a single homomeric or heteromeric receptor could bias signaling to one G protein versus another. Using a combination of chimeric G proteins, one could determine the ligand selectivity for G protein coupling.

3.5 Results Analysis

1. The assay records, from the designated wells, a pre-assay endpoint (to determine basal fluorescence), emitted fluorescence by column, and then a post-assay endpoint. For data analysis, it is especially important to record the pre-assay endpoint (see Note 18).
2. Data can be saved as SoftMax profile (.pda) or exported as text file, which can be opened in a spreadsheet program (e.g., Microsoft Excel, Open Office spreadsheet).
3. The SoftMax pro datasheet provides the pre- and post-assay endpoints, each time point, and a normalized value of the peak value (peak – basal).

4. Data can be represented as $[(\text{peak} - \text{basal})/\text{basal}] \times 1,000$ or as area under the curve (AUC, Fig. 5). The pre-assay endpoint is the basal value, while the set of numbers just after the last column analysis and just before the post-assay endpoint are the peak values. The maximum peak height, the AUC, and the shape of the calcium response may all be informative (see Note 19).

4 Notes

1. A different chimeric G protein (3) or no G protein at all can be transfected here, depending on the type of receptor used and the output to be investigated (for example, if a heteromeric receptor is postulated to have a novel G_q -protein coupling different from its monomers, then no G protein may be transfected).
2. Depending on the cell type, cells can be counted using either a hemacytometer or a machine such as a NucleoCounter (New Brunswick Scientific, Edison, NJ).
3. This concentration gives 70,000 cells/well. The FLIPR protocol recommends seeding 50,000 cells/well, but we adjust this number depending on cell type and incubation timeframe.
4. The 96-well plate can be pretreated with poly-d-lysine (PDL) to prevent cells from detaching easily during handling. PDL pretreatment becomes especially important when a relatively harsh method of transfection is used (e.g., cells tolerate FuGENE 6 (Roche) better than Lipofectamine). Additionally, cells should preferably be transferred quickly using a multi-channel pipette or a multidrop dispenser to maintain a homogeneous solution. Otherwise, resuspend the cell solution every couple of minutes.
5. Preferably a cell line stably expressing the receptor of choice (and chimeric G protein) should be used. This will reduce the variation in expression levels that arises from transient transfections. This variation becomes a bigger issue when co-expressing two receptors and looking for novel functions on putative heteromers. In this case at least three cell lines should be generated: one expressing receptor A alone, one expressing receptor B alone, and one expressing both receptor A and receptor B at the same levels as in the homomer cell lines, so that a direct comparison of potency and efficacy can be made (for example, if homomer line A has 50 fmol/mg of receptor A but the heteromer line has 200 fmol/mg of receptor A, both efficacy and potency may be altered, since receptors are competing for transfected G protein as well as endogenous G protein). However, generating heteromer cell lines with expression levels matched to existing homomer lines may not always result in an optimal ratio of heteromer formation. Thus, it is advisable to generate multiple heteromer cell lines with varying ratios of homomers and heteromers, and then select matching homomer cell lines. Therefore, to insure success with this assay, it is best to establish and fully characterize homomer and heteromer cell lines prior to beginning a screen for novel activity on heteromers. The ratio of homomers and heteromers can be determined by several methods including serial co-immunoprecipitation or FRET/BRET analysis.

6. Other transfection methods include FuGENE 6 (Roche), which is gentler to the cells, or calcium phosphate, which is relatively inexpensive and works well with HEK-293 cells.
7. To speed up aspiration a multichannel aspirator head can be used (e.g., multiwell plate washer/dispenser manifold, Sigma).
8. At this stage pertussis toxin (PTX) or cholera toxin (CTX) can be added to the medium. PTX catalyzes the ADP-ribosylation of the α -subunits of the heterotrimeric G proteins G_i , G_o , and G_t , preventing the G proteins from interacting with the receptor. Similarly CTX permanently ribosylates the G_s alpha subunit. The use of the PTX and/or CTX may reveal that a GPCR or heteromer can couple to multiple G proteins, or increase signal output by forcing a GPCR to couple to, for example, a chimeric PTX insensitive G protein (10).
9. The FLIPR assay kit comes equipped with assay buffer. However, the amount provided in the kit may not be sufficient if multiple agonist and antagonist dilution series are prepared. We make additional buffer as follows: For 100 ml, use 10 ml of 10 $\times$ HBSS, 2 ml 1 M HEPES, 1 ml 0.25 M probenecid [made fresh and dissolved in 1 M NaOH], and 87 ml dH₂O).
10. To avoid dislodging cells from the 96-well plate, pipette gently at a 45° angle and avoid touching the bottom of the wells.
11. Since the calcium release occurs in a matter of seconds, the agonist should always be added by the FlexStation.
12. The depth of the pipette tip, the speed, and the volume of transfer can all have an impact on how well the cells remain fixed to the bottom of the well. For a volume of 200 μ l a pipette height of 225 μ l and a transfer rate of 3 can be used.
13. When placing the ligand plate and assay plate (cell plate) in the FlexStation, make sure they are correctly oriented (i.e., A1 for both plates is in the upper left corner).
14. In order to conserve ligand, all volumes can be reduced by half, and the ligands prepared at a 3 $\times$ concentration (the assay is then run using 50 μ l cells, 50 μ l ligand, and 50 μ l assay/fluorophore buffer). In practice, this requires 100 μ l of ligand per well (The machine may not reliably transfer the desired volume if the well is filled with less than 100 μ l). The cells are transfected in the same manner (i.e., placed in 100 μ l medium), but before addition of the FLIPR reagent to the cells, 50 μ l of buffer is removed from each well of the cell plate. Then 50 μ l of FLIPR reagent is added to the cells to give a 100 μ l solution to which the 50 μ l of 3 $\times$ ligand will be added. Make sure to change the “compound transfer” conditions under setup to 100 μ l initial volume and 125 μ l pipette height.
15. The solvent may have an impact on the readout. As seen from Fig. 6, concentrations of 5% DMSO, ethanol, and methanol should be avoided. However, we found that mildly acidic (pH 3, 1 mM HCl) or alkaline solutions (pH 11, 1 mM NaOH) had no significant effect on basal Ca²⁺ mobilization.

16. While 50 μ l of ligand/buffer will be transferred from the reagent plate to the assay plate, depending on the plate used (flat bottom, round bottom, v-bottom), in order to make sure the FlexStation can access the solution, more than 50 μ l ligand solution will be needed in each well. In general, try to prepare at least 100 μ l volume for each concentration.
17. When pre-adding the antagonist, add to each column in timed intervals (e.g., 2 min, using a multichannel pipette) in order to keep the actual preincubation time in line with the time it takes to measure one column in the FlexStation. If the antagonist is added separately, make sure to adjust the concentration of both the antagonist as well as the agonist to the final volume during measurement: 100 μ l medium, 100 μ l dye, 50 μ l antagonist, and 50 μ l agonist require the antagonist and agonist concentrations to be six times more concentrated. Alternatively 25 μ l of antagonist and agonist can be used (10 $\times$ more concentrated).
18. The “pre-assay endpoint” window must be selected when the assay has begun in order for this endpoint to be recorded. Additionally, the empty data file must be saved (as well as the protocol) both before and after the assay. Otherwise the FlexStation may not measure the pre-assay endpoint.
19. The temporal and spatial activity of Ca^{2+} is also known as a Ca^{2+} -wave. The Ca^{2+} -wave relies on release of Ca^{2+} from intra-cellular stores (via activation of IP_3 and ryanodine receptors) or the influx of Ca^{2+} across the plasma membrane through Ca^{2+} channels. To only study Ca^{2+} mobilization from intracellular stores, cells can be grown in Ca^{2+} -free medium or in the presence of Ca^{2+} chelators such as EGTA. On the other hand, the intra-cellular Ca^{2+} stores in the endoplasmatic reticulum can be depleted by administration of thapsigargin. Similarly, dantrolene and ryanodine can block ryanodine receptors and limit the passage of Ca^{2+} from smooth ER. The origin of Ca^{2+} release and propagation of a Ca^{2+} -wave may be of interest when studying GPCRs involved in, for example, neurotransmitter release.

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Fig. 1.

Measuring Ca^{2+} mobilization by activation of a G_q -, G_i -, or G_s -coupled receptor. HEK-293 cells were transfected with the G_q -coupled cholecystokinin CCK2 receptor, the G_i -coupled mu-opioid receptor (with or without the chimeric G_{qi4} -protein) or the G_s -coupled dopamine D_1 receptor (with or without the chimeric G_{qs4} -protein). Calcium mobilization was measured using the FLIPR assay kit, after activation of the receptors by an appropriate agonist (CCK-8S, etorphine, and dopamine, respectively)

Fig. 2. Heteromer ligands. Monovalent ligand 6'-GNTI and the MDAN bivalent ligand series ($n = 5$, MDAN-19)

Fig. 3. Measuring antagonism using FLIPR. Increasing doses of DOR antagonist BNTX decrease the potency of DOR agonist DPDPE. Antagonist was incubated with cells for 10 min prior to assay

Fig. 4.

(a) 6'-GNTI-induced Ca^{2+} release in HEK-293 cells expressing one or two opioid receptor types. Agonist-mediated intracellular Ca^{2+} release was measured in cells expressing the chimeric G protein 6-G_{qi4-myr} (200 ng for every 40,000 cells). (Reproduced from ref. 8 with permission. Copyright 2005 National Academy of Sciences, USA.) (b) Effects of receptor-type-selective antagonists on 6'-GNTI-induced Ca^{2+} release in cells expressing KOR/DOR heteromers. Cells were preincubated for 30 min with increasing doses of NTI or NorBNI and stimulated with 100 nM 6'-GNTI (Reproduced from ref. 8 with permission Copyright 2005 National Academy of Sciences, USA.)

Fig. 5.

Agonist-induced calcium mobilization: The calcium wave. Calcium mobilization can be represented as $[(\text{peak} - \text{basal})/\text{basal}] \times 1,000$ or as area under the curve (AUC)

Fig. 6. Effect of DMSO, ethanol, and methanol concentration on calcium mobilization in the FLIPR assay. Ca^{2+} mobilization was measured in HEK-293 cells using the FLIPR assay kit. Cells were stimulated with increasing concentrations of DMSO, ethanol, or methanol to a final concentration of 5% (v/v). The *solid line* represents response to buffer