Chapter 8

Reporter Gene Assays

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Summary

Reporter gene assays are versatile and sensitive methods of assaying numerous targets in high-throughput drug-screening programs. A variety of reporter genes allow users a choice of signal that can be tailored to the required sensitivity, the available detection apparatus, the cellular system employed, and the required compatibility with multiplexed assays. Promoters used to drive reporter gene expression can be activated either by a broad range of biochemical pathways or by the selective activation of individual targets. In this chapter, we will introduce some of the considerations behind the choice of reporter gene assays and describe the methods that we have used to establish 96-well format luciferase and aequorin assays for the screening of ligands for G protein-coupled receptors.

Key words: Aequorin, Chimera, CRE, GPCR, G-protein, Luciferase, NF-kappa-B, Receptor, Reporter gene, STAT.

1. Introduction

The development of cell-based high-throughput screening platforms owes its success to the ability to manipulate the genetic and biochemical composition of eukaryotic cells. Notably, cells can be engineered to express specific gene products in response to a given stimulus. The gene product itself may possess an inherent property that enables it to be measured directly, e.g., green fluorescent protein (GFP), or it may display enzymatic activity that can be monitored, e.g., luciferase. Alternatively, the gene product may respond to changes in the levels of a signaling molecule, e.g., the Ca^{2+} ion-mediated activation of aequorin luminescence. The continuing development of genetically engineered vectors, coupled with powerful detection techniques, allows many cell types

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to be engineered as biodetectors for myriad classes of biochemical and signaling pathways.

The reporter gene unit consists of a promoter and the reporter gene. When establishing an assay system, a number of factors need to be considered in order to optimize the assay. The choice of promoter, the number of copies of the promoter, and the nature of the reporter gene will allow control of the basal level of reporter gene activity, control of the specificity of activation, and control of the degree of stimulation measured *(1)*. Endogenous promoters, such as c-fos, the cAMP response element (CRE), or the estrogen response element, are commonly used, but may suffer interference from endogenous intracellular signaling events. However, their regulation by multiple signaling pathways makes them widely applicable, even allowing drugs that activate different signaling events to be identified in the same assay format. Alternatively, exogenous promoters, such as the yeast Gal4 response element system, can be used to reduce unwanted activation by native transcription factors *(2)*. There are many examples of the optimization and employment of a variety of promoters in high-throughput screening programs *(3)*.

The reporter gene itself should ultimately generate a signal that can be clearly identified. The reporter gene products can be either intracellular or extracellular in nature. Intracellular products are retained in the cell for quantification in situ or following cell lysis. Extracellular products are secreted into the extracellular medium for assay, allowing repeated experimentation and sampling without disturbing the cells. Commonly used intracellular reporter genes are chloramphenicol acetyltransferase (CAT), β-galactosidase, luciferase, aequorin, and GFP. Extracellular reporter genes are usually secreted placental alkaline phosphatase (SPAP) or β-lactamase *[(3)* and references therein]. Each reporter gene has its advantages and disadvantages in terms of sensitivity, time taken for signal generation, ease of signal detection (e.g., products can be radioactive, fluorescent, bioluminescent, etc.), cost, miniaturization, and automation. A further consideration may also be the compatibility within dual reporter gene assays or multiplexed assays.

A recent report of a dual reporter gene assay demonstrated the ease with which signals from two different classes of G protein-coupled receptors (GPCRs) can be measured simultaneously. Two cell lines were generated that expressed either CRE coupled to firefly luciferase (CRE-luc) or to a 12-*O*-tetradecanoyl-phorbol -13-acetate (TPA)-response element coupled to *Renilla* luciferase. Coseeding of the reporter cell lines allowed independent or simultaneous measurement of ligand activation of GPCRs that activate distinct intracellular signaling pathways *(4)*. The authors have also suggested that the inclusion of a third GFP-based reporter

construct would allow three separate GPCRs to be coassayed. Reporter gene assays have also proven amenable to inclusion in multiplexed assays that also measure Ca^{2+} mobilization in a 384-well format. For example, by using the β-lactamase-based GeneBLAzer® reporter gene system (Invitrogen, Carlsbad, CA) and loading the same cells with the calcium-sensing Fluo-4 NW dye, researchers were able to rapidly verify positive *hits* in a drug screen by correlating the data from Ca^{2+}-based assays and reporter gene assays performed sequentially on the same cells *(5)*. Running these two assays also facilitated the rapid elimination of false positives generated by either assay format, allowing huge savings in cost and time to be made.

In our experience, reporter gene-based assays are an effective way for laboratories with limited resources to develop and deploy innovative and sensitive high-throughput screening programs. Reporter gene assays can be applied to a huge range of targets including GPCRs, nuclear receptors, receptor tyrosine kinases, enzymes, and transcription factors. Furthermore, by tailoring the incorporation of relevant promoters and reporter genes into different assays, diverse screening programs can be established that utilize common detection equipment, increasing the cost efficiency of drug screening.

We use both luciferase and aequorin reporter genes. The luciferase genes are driven by STAT3, NFκB, or CRE promoters. These allow us to assay most GPCRs, with G_s- and $G_{i/o}$-coupled GPCRs effectively regulating CRE-driven luciferase activity by modulating intracellular cAMP levels *(6)*. G_q- and $G_{i/o}$-coupled GPCRs are assayed by either STAT3- or NFκB-driven assays, while $G_{12/13}$-coupled receptors generally promote NFκB-driven luciferase activity *(7–9)*. The flexibility of all of our reporter gene assays is greatly enhanced by the incorporation of promiscuous or chimeric G protein α-subunits, notably $G\alpha_{16}$, 16z25, or 16z44 *(10)*. These subunits allow virtually all GPCRs tested to date to promote the activation of phospholipase Cβ (PLCβ)/inositol trisphosphate (IP_3)/Ca^{2+} pathways, enabling a single assay to be used to screen for GPCR activity. Numerous chimeras have been stably or transiently expressed in a variety of cell lines and incorporated into a number of other assay platforms, including FLIPR, microarrayed compound screening, and yeast autocrine selection *(11, 12)*. In fact, cell lines stably expressing GPCRs and promiscuous G proteins are commercially available (Chemicon, Temecula, CA). Using endogenous and chimeric G proteins, we and others have successfully incorporated dozens of GPCRs into aequorin-based reporter gene screening programs *(13–15)*. In this chapter, we will detail the methods that we regularly use in our own GPCR analysis and screening programs.

2. Materials

1. Dulbecco's Modified Eagle Medium (DMEM) (Gibco/Invitrogen/Invitrogen, Carlsbad, CA).
2. Modified Eagle Medium (MEM) (Gibco/Invitrogen).
3. OPTI-Modified Eagle Medium (Opti-MEM, Gibco/Invitrogen).
4. Phenol red-free DMEM (Sigma-Aldrich; St. Louis, MO).
5. Fetal bovine serum (FBS; Gibco/Invitrogen).
6. Dulbecco's phosphate-buffered saline (PBS; Gibco/Invitrogen).
7. Hanks' balanced salt solution (HBSS), with or without calcium chloride (Gibco/Invitrogen).
8. Solution of trypsin (1×) is diluted from trypsin (10×) with PBS (Gibco/Invitrogen).
9. Solution of penicillin/streptomycin (20×) (Gibco/Invitrogen).
10. Zeocin (250mg/mL) (Gibco/Invitrogen).
11. Geneticin (G418; Calbiochem, San Diego, CA) is dissolved in Opti-MEM at 100mg/mL and stored at –20°C. The working concentration is 50mg/mL.
12. Lipofectamine PLUS and Lipofectamine 2000 reagents (Gibco/Invitrogen).
13. 2× HEPES-buffered saline (HBS): 280mM NaCl, 10mM KCl, 1.5mM Na_2HPO_4, 12mM dextrose, and 50mM HEPES (pH 7.05). The solution should be sterilized by passing through a 0.2-μm filter.
14. Multichannel pipettor, Multipette Plus dispenser and Combitips Plus (5mL and 2.5mL) (Eppendorf, Westbury, NY).
15. Tissue-treated 96-well polystyrene white plates with flat bottoms (Nalge Nunc, Rochester, NY).
16. Polypropylene 96-well round-bottom block and solvent reservoir (USA Scientific, Ocala, FL).
17. Conical 96-well polypropylene plates (Corning Costar, Lowell, MA).
18. Mitochondrial apoaequorin expression vector and coelenterzine *f* (Invitrogen).
19. Luciferase reporter gene constructs with STAT3-, CREB-, and NFκB-response elements (pSTAT3-Luc, pCRE-Luc, and pNFkB-Luc; Clontech, Mountain View, CA).

20. Luciferin and luciferase lysis buffer are from a luciferase assay kit (Roche, Mannheim, Germany).
21. 96-well format luminometer (EG&G Berthold, Bundoora, Australia).

3. Methods

3.1 Luciferase Assay

3.1.1. Preparation of Cells Stably Expressing CRE- or NFκB-Driven Luciferase Reporter Genes

To minimize the variations in different sets of experiments run on different days, stable cell lines carrying CRE- or NFκB-driven luciferase reporter genes have been generated. The plasmids carrying these reporter genes do not encode any selection markers. Therefore, we cotransfect pcDNA3 to provide a means to select cells carrying both the reporter gene constructs and pcDNA3. We have found that it is not necessary to isolate a clonal cell line. Cells that survive the selection process are harvested and provide robust, consistent responses almost indefinitely. After the establishment of the stable cell lines, cDNAs encoding GPCRs and G proteins can be transiently introduced into the cells prior to the assays (**Subheading 3.1.2**).

1. Seed 100-mm plates of 2×10^6 HEK293 cells in MEM medium containing 10% FBS and 1× penicillin/streptomycin (*see* **Note 1**).
2. The following day, the medium should be exchanged for 9-mL fresh MEM/10% FBS/1× penicillin/streptomycin.
3. After 3h, prepare calcium phosphate transfection buffers as described here (the indicated volumes are required for each plate of cells):
   (a) Aliquot 500μL 2× HBS into a 5-mL sterile plastic tube.
   (b) Prepare the DNA mix in a second tube containing 50μL of 2.5M $CaCl_2$ (sterilized through a 0.2-μm filter), 9μg of reporter gene cDNA, and 1μg of selection marker DNA (pcDNA3), and make up volume to 500μL with H_2O.
   (c) Add DNA mix slowly to 2× HBS by pipetting dropwise.
   (d) Use a pipette to bubble air though the solution for (30 seconds) to allow complete mixing.
   (e) Incubate at room temperature for 20min.
   (f) Add the mix to the cells dropwise with gentle swirling.
4. Incubate for 16h, 37°C, 5% CO_2.
5. Aspirate the transfection medium, wash transfected cells with PBS three times to completely remove calcium phosphate-DNA

precipitates, and replenish with fresh MEM/10% FBS/1× penicillin/streptomycin.

6. Incubate at 37°C, 5% CO_2 overnight.
7. Aliquot 50µL of 100mg/mL G418 to each 100-mm plate for selection, for a final concentration of 500µg/mL G418.
8. Fresh medium with 50µL of 100mg/mL G418 (to give a concentration of 500µg/mL) is replenished every 3–4 days, after washing three times with PBS, until selection is finished (*see* **Note 2**).
9. Cells stably carrying luciferase reporter are maintained in 250µg/mL G418.

*3.1.2. Transient Transfection of GPCR and G Protein cDNAs into Cell Lines Stably Carrying Luciferase Reporter Gene Constructs (see **Note 3**)*

1. 15,000 HEK293 cells carrying luciferase reporter genes (*see* **Subheading 3.1.1**) are seeded per well of a 96-well white microplate using MEM (10% FBS).
2. The following day prepare the transfection cocktail as described here:
   (a) Mix 12.5-ng GPCR cDNA and 37.5-ng G protein cDNA per well.
   (b) Dilute 0.2µL of PLUS reagent with 25-µL Opti-MEM per well.
   (c) Add PLUS/Opti-MEM mixture to the cDNA.
   (d) Incubate at room temperature for 15min.
   (e) Dilute 0.2-µL Lipofectamine reagent with 25-µL Opti-MEM per well.
   (f) Add the diluted Lipofectamine to the cDNA/PLUS/Opti-MEM solution.
   (g) Incubate at room temperature for a further 15min.
3. Remove the growth medium from the white microplate seeded with the reporter stable cell line.
4. Aliquot 50µL of the transfection cocktail to each well of the white microplate.
5. Incubate at 37°C for 3h.
6. Add 25µL of Opti-MEM containing 30% FBS.
7. Further incubate at 37°C overnight.
8. Proceed to **Subheading 3.1.4** for assay.

*3.1.3. Preparation of Cells Transiently Expressing STAT3 Promoter-Driven Luciferase Reporter Gene Constructs (see **Note 3**)*

In contrast to the CRE promoter- and NFκB promoter-driven luciferase reporter genes, attempts to establish HEK293 cell lines stably expressing STAT3 promoter-driven luciferase reporter genes have resulted in luciferase signals that diminish with each passage of the cells. Therefore, transient introduction of the

STAT3-driven luciferase reporter gene, along side the GPCR and G protein cDNAs, is employed.

1. 15,000 HEK293 cells are seeded per well in a 96-well white microplate with MEM (10% FBS).
2. The following day, prepare the transfection cocktail following **step 2** of **Subheading 3.1.2**, with a modification to **step 2a**.
   (a) Mix 10-ng GPCR cDNA, 10-ng G protein cDNA, and 100-ng STAT3 reporter cDNA per well.
3. Remove growth medium from the white microplate seeded with HEK293 cells.
4. Distribute 50-μL Opti-MEM to each well.
5. Aliquot 50μL of the transfection cocktail to each well.
6. Incubate at 37°C for 3h.
7. Add 25μL of Opti-MEM containing 30% FBS.
8. Further incubate at 37°C overnight.
9. Proceed to **Subheading 3.1.4** for assay.

3.1.4. Ligand Induction

Ligand Induction for NFκB- and STAT3-Luciferase Assays (*see* ***Note 4***)

1. Prepare and dilute appropriate agonists and/or antagonists using phenol red-free DMEM (50μL will be required for each well of a 96-well plate).
2. Remove the transfection cocktail from the 96-well white microplate.
3. Aliquot 50μL of the prepared solutions with ligands or phenol red-free DMEM medium (as a basal control) to appropriate wells. For determination of the contribution of $G\alpha_{i/o}$ proteins to a response, wells should be pretreated with 100ng/mL PTX (in phenol red-free DMEM) for 4h before ligand addition. The PTX can be added directly to the transfection cocktail.
4. Incubate at 37°C for 16h.
5. Remove the medium completely.
6. Aliquot 25-μL luciferase lysis buffer per well and store at –80°C until use in the luciferase assay (*see* **Subheading 3.1.5**).

Agonist Induction for CRE Assays (*see* ***Note 4***)

1. Prepare diluted ligands and controls using phenol red-free DMEM.
2. Remove the transfection cocktail from the 96-well white microplate.
3. Aliquot 50μL of the prepared ligand solutions or controls, as appropriate, to the wells. Should forskolin be required, it should also be included at this point at a final concentration of 10μM (*see* **Note 5**).

4. Incubate for:
   (a) To measure activation of adenylyl cyclase by G_s-coupled GPCRs: overnight at 37°C.
   (b) To measure inhibition of adenylyl cyclase activity by $G_{i/o}$-coupled receptors: 30min at 37°C. Remove stimulus, including forskolin, completely and wash twice with 100-µL phenol red-free DMEM. Replenish with 50-µL phenol red-free MEM and incubate at 37°C for 16h.
5. Remove medium completely.
6. Aliquot 25-µL luciferase lysis buffer per well and store at –80°C until use in the luciferase assays (*see* **Subheading 3.1.5**).

*3.1.5. Luciferase Assay (see **Note 6**)*

1. Prepare luciferin solution and 1× luciferase lysis buffer, and equilibrate both to room temperature (25µL of each solution will be required per well).
2. Prewarm the luminometer to 25°C. Ensure that the 96-well plates have been removed from –80°C and thawed.
3. Charge injector P, of the 96-well luminometer (EG&G Berthold, Bundoora, Australia), with luciferin solution and injector M with luciferase lysis buffer.
4. Inject 25-µL luciferase lysis buffer by injector M followed by 25-µL luciferin solution from Injector P to each well of the luminescent white plate.
5. Monitor luminescence released for 10s after luciferin addition. Examples of the data generated by these luciferase reporter assays can be seen in **Figs. 1** and **2**.

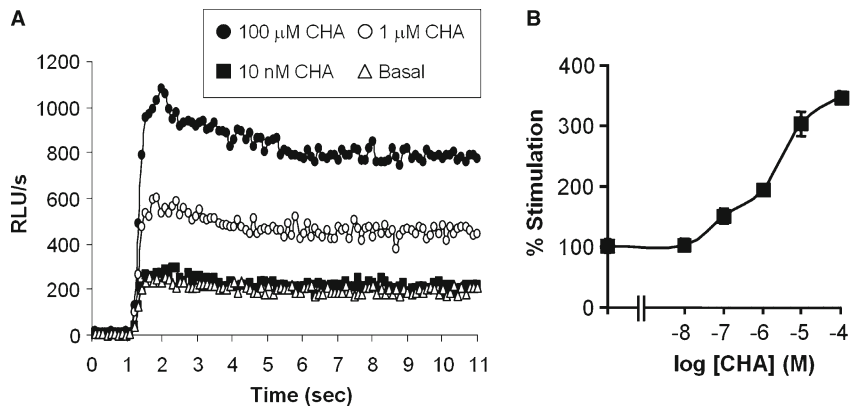

Fig. 1. Dose-response curve of N^6-cyclohexyladenosine (CHA, selective agonist for adenosine A_1 receptor) for A_1R/$G\alpha_{16}$-transfected HEK293 cells stably carrying a NFκB-driven luciferase reporter construct. To eliminate $G_{i/o}$ signal transduction, transfectants were pretreated with pertussis toxin (100 ng/mL) for 4 h prior to agonist induction. Different concentrations of CHA were added to the cells for 16 h, and the luciferase activities of the cell lysates were measured. (**A**) Triplicates were performed and the results from one set of wells are illustrated as representative. (**B**) Construction of the dose-response curve was accomplished by plotting the % stimulation corrected to the basal vs. the logarithmic concentrations of CHA. Data shown represent the mean ± SE performed in triplicate.

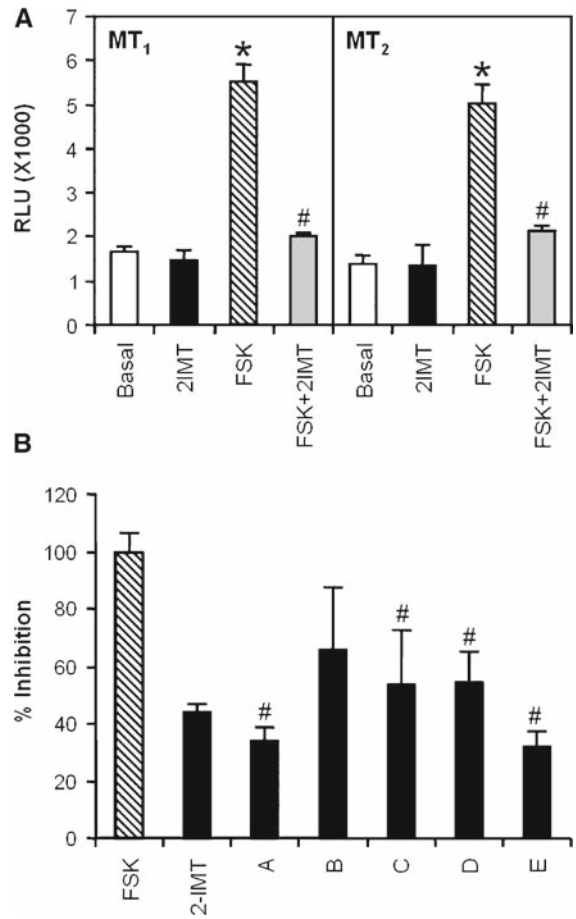

Fig. 2. The inhibitory responses of melatonin type 1 and type 2 receptors (MT_1 and MT_2) coupled to $G_{i/o}$ proteins as measured by a CRE-driven reporter gene. HEK293 cells stably carrying a CRE-driven luciferase reporter construct were transfected with MT_1 or MT_2 and the transfectants were challenged with plain medium (basal), 2-iodomelatonin (an agonist for MT_1 and MT_2; 2-IMT, 10 μM), forskolin (FSK, 10 μM), or FSK with 2-IMT. (**A**) Raw integrated RLUs were plotted and data shown represent the mean ± SE performed in triplicate. (**B**) A panel of 2-IMT derivatives (A–E) was exposed to MT_1-transfected HEK293 cells stably carrying the CRE-driven luciferase reporter. The magnitudes of % inhibition of the forskolin response (where FSK stimulation alone was set to 100%) were plotted and data shown represent the mean ± SE performed in triplicate. *, agonist-induced RLU is significantly higher than the basal level (Dunnett's *t* test, $p < 0.05$); #, agonist-induced response was significantly inhibited as compared to FSK (Dunnett's *t* test, $p < 0.05$).

3.2. Aequorin Screening

This screening is primarily made possible by the coupling of GPCRs to G proteins, leading to downstream PLCβ activation, IP_3 accumulation, and Ca^{2+} release from endoplasmic reticulum. The released Ca^{2+} is quantified by the presence of Ca^{2+}-sensitive aequorin photoprotein. The aequorin complex is composed of apoaequorin, coelenterazine (a cofactor), and oxygen,

which readily reacts with Ca^{2+}, causing the degradation of the coelenterazine-bound aequorin, generating coelenteramide and luminescent flashes. Both transient and stable transfections of the apoaequorin have been tested, but we find that the response generated from the transient introduction of apoaequorin gives better signal-to-noise ratios.

*3.2.1. Transient Transfection (see **Note 3**)*

1. COS-7 cells are seeded at 10,000 cells/well using DMEM (10% FBS) in 96-well white luminescent plates.
2. The following day, prepare transfection cocktail as described here:
   (a) Mix 100-ng apoaequorin cDNA, 50-ng GPCR, and G protein cDNAs per well.
   (b) For each well, dilute 0.2μL of PLUS reagent with 25-μL Opti-MEM.
   (c) Add the PLUS/Opti-MEM diluent to the cDNA mix.
   (d) Incubate at room temperature for 15min.
   (e) Dilute 0.2μL of Lipofectamine reagent with 25-μL Opti-MEM per well.
   (f) Add the diluted Lipofectamine to the cDNA/PLUS/Opti-MEM mix.
   (g) Incubate at room temperature for a further 15min.
3. Remove growth medium from the 96-well microplate.
4. Aliquot 50μL of the transfection cocktail to each well of the microplate.
5. Incubate at 37°C for 3h.
6. Add 50-μL Opti-MEM containing 4% FBS (*see* **Note 7**).
7. Incubate at 37°C for 48h.

*3.2.2. Apoaequorin Labeling (see **Note 8**)*

1. For each well prepare labeling medium consisting of 100-μL calcium-free HBSS (pH 7.5) with 2.5μM coelenterazine *f* and 20mM HEPES (pH 7.4).
2. The transfection medium is removed from the 96-well microplate and replaced by 100μL of the labeling medium.
3. Place the microplate in a dark, humidified incubator at 37°C for 4h.

*3.2.3. Aequorin Assay (see **Note 9**)*

1. The luminometer should be prewarmed to 37°C.
2. Prepare appropriate controls and drug solutions in 3× concentrations (50μL per well will be required).
3. Warm the drug solutions to 37°C.
4. Set Injector P to inject 50-μL control or drug solutions.
5. Following injection, monitor the luminescence released for 15s. Examples of the types of data generated by aequorin assays can be seen in **Figs. 3** and **4**.

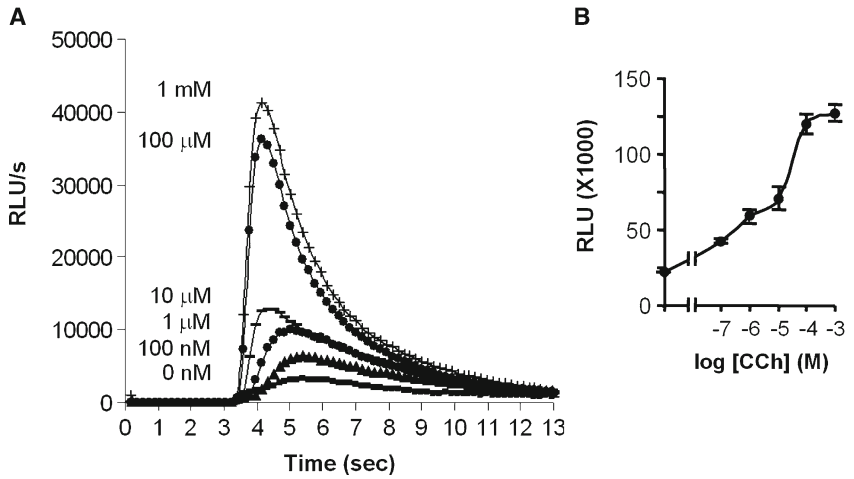

Fig. 3. Dose-response curves of carbachol for COS-7 cells transiently transfected with muscarinic type 1 receptor (M_1R) and apoaequorin. Transfectants were labeled with 2.5 μM coelenterazine *f* for 4 h. (**A**) Serial dilutions of carbachol were prepared and screening was performed in increasing order of carbachol concentrations. Triplicates were performed and the results from one set of wells are illustrated as representative. (**B**) Construction of a dose-response curve was accomplished by plotting the total integrated RLUs vs. the logarithmic concentrations of carbachol. Data shown represent the mean ± SE performed in triplicate.

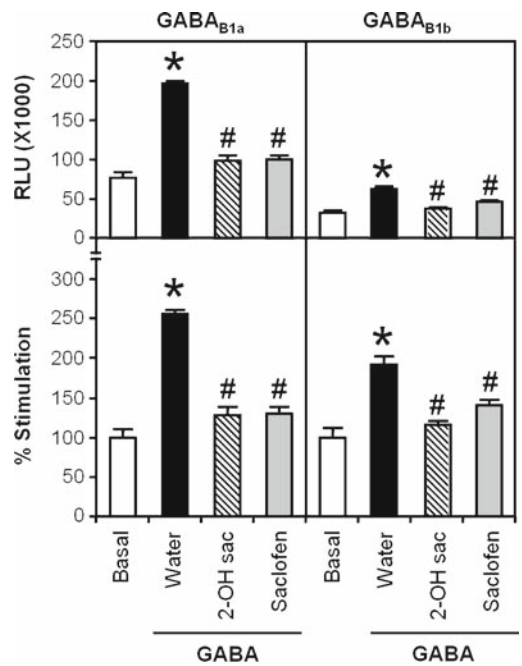

Fig. 4. Aequorin assay of the effects of $GABA_B$-selective antagonists in COS-7 cells transfected with $GABA_{B2}$ and either $GABA_{B1a}$ or $GABA_{B1b}$ receptor subunits, as well as the G protein α-subunit 16z25. Transfectants were treated with basal (*open bar*), GABA (500 μM; *closed bar*), GABA with 2-hydroxy saclofen (2-OH Sac; 50 μM; *diagonally striped bar*), and GABA with saclofen (50 μM, *gray bar*). 2-OH Sac and saclofen are antagonists at $GABA_B$ receptors and they were added simultaneously with GABA. Raw integrated RLUs and % stimulation (as compared to their corresponding basals) were plotted. Data shown represent the mean ± SE performed in triplicate. *Agonist-induced RLU is significantly higher than the basal level (Dunnett's t test, $p < 0.05$); #, antagonist significantly suppressed the GABA-induced Ca^{2+} mobilization (Dunnett's t test, $p < 0.05$).

3.3. Data Analysis

WinGlow software is supplied with the luminometer. This software, in addition to recording the experimental parameters used, allows direct data transfer to Microsoft Excel as Macros for analysis. Data is exported as a time course (both before and after the stimulus injection) of the relative luminescent unit (RLU) released. The readouts in Excel include the peak value, the time when the peak value is reached, and the integrated area under the curve in terms of RLU. For both luciferase and aequorin assays, to ensure better reproducibility within assays, we prefer to rely on integrated areas beneath the curves, i.e., 10s for luciferase or 15s for aequorin assays. To further improve the consistency between assays performed on different days, other standards are useful. The first common standard used is the introduction and normalization of the response to an empty vector control. In other cases, for instance, when the efficacy of an agonist is to be evaluated, the fold-stimulation or percent-stimulation over a blank control is used.

4. Notes

1. Transfections may be done in duplicate. Plates should also be seeded for mock transfections (i.e., cells transfected in the absence of cDNA) to allow for determination of the endpoint of the selection procedure (*see* **Note 2**). For calcium phosphate transfection of HEK293 cells, cells should be seeded at approximately 40–60% density. Seeding cells just before the day of transfection avoids crowding of cells, which may lower the transfection efficiency. The pH of the HBS and the incubation time with the transfection cocktail play significant roles in the transfection efficiency. Incubation time periods normally range from 8h to a maximum of 16h. Prolonged incubation of cells with the calcium phosphate precipitate induces cell death. In addition, after incubation with the transfection cocktail, it is crucial to thoroughly rinse the cells with PBS to completely remove the calcium phosphate; however, care should be taken not to wash the cells from the plate.
2. Cell death starts to be observable after G418 has been introduced for the first 4–5 days. Cells should be washed with PBS and new G418 should be replenished into the fresh medium every few days. Normally, the whole selection process takes 2–3 weeks to finish, at which point all cells in the mock transfected plates should be dead and any living cells in the transfected plates can be assumed to be stably expressing the reporter gene constructs.

3. Triplicates are always performed in our 96-well format assays, and often many 96-well plates are transfected with the same cDNAs. Hence, instead of mixing cDNAs for individual wells, *master mixes* for a number of wells are prepared to reduce the number of pipetting steps and possible pipetting errors. A multichannel pipettor is employed to ease the process of removing growth medium and adding transfection cocktail to microplates. Complete removal of the growth medium before addition of the transfection cocktail is essential, as serum in the medium hinders the transfection by LipofectaminePLUS reagents.
4. As phenol red has been shown to have an effect on the luciferin reaction, phenol red-free medium is used for drug treatment after transfection. In addition, the transfection cocktail must be thoroughly removed to avoid activation of the response elements by growth factors and hormones, among other things, in the serum. At least one freeze–thaw cycle is required for complete cell lysis by the luciferase lysis buffer. Thus, the collected cell lysates can be kept at –80°C until required for the luciferase assays.
5. For the CRE-driven luciferase reporter, two different experimental designs are optimized for the evaluation of either the activation or inhibition of adenylyl cyclase through the regulation of G_s or $G_{i/o}$ proteins, respectively. For the activation of G_s proteins, the experimental setup is similar to that for STAT3- or NFκB-driven luciferase assays. However, for measurements of $G_{i/o}$ protein-mediated activity, the activation of $G_{i/o}$ proteins is revealed through their inhibition of adenylyl cyclase activity. Adenylyl cyclase can be activated either directly by forskolin or through the activation of G_s proteins by a G_s-coupled GPCR. In most of our studies, forskolin is used, as it guarantees robust stimulation of adenylyl cyclase. This necessitates the use of extra controls. Generally, we use basal agonist for the receptor under investigation, forskolin alone, and forskolin with the receptor's agonist. The washing step is particularly important in CRE-driven luciferase assays using forskolin. Traces of forskolin induce remarkable CRE-driven luciferase activity and overwhelm the inhibitory effects mediated by the $G_{i/o}$ proteins. Complete removal of residual forskolin is accomplished by careful aspiration of stimuli and additional washing steps with plain medium. Alternatively, the activation of a G_s-mediated pathway can be achieved by cotransfection of a G_s-coupled receptor. This G_s-mediated activation of adenylyl cyclase is generally weaker than the forskolin-mediated stimulation, and may be preferred in the examination of G_i-coupled receptors that couple weakly to adenylyl cyclase.

6. To maintain consistency between runs, the luciferin substrate solution, the lysis buffer, and the microplate reader are always prewarmed to ambient temperature before assays. An additional 25-μL lysis buffer is injected to ensure that the samples of cell lysates are equilibrated to ambient temperature and the cell lysate is evenly mixed and distributed throughout the well.
7. The growth rate for COS-7 cells is faster than for HEK293 cells; thus, a final concentration of 2% fetal bovine serum is used for the replenishment after transfection.
8. Activated aequorin, i.e., coelenterazin *f*-bound apoaequorin, reacts quickly with Ca^{2+} for luminescence release. Therefore, it is absolutely essential to maintain a Ca^{2+}-free environment starting from the labeling step.
9. Since intact cells are used for the aequorin assays, the residing platform inside the luminometer, the microplate, and the plastic tubes with the drug solutions are warmed to 37°C. A sudden temperature change for charged cells could cause stress and induce inaccurate readings. Since only one injector (Injector P) is available and suitable in MicroLumat (Berthold) for drug distribution, the screening itself always starts with wells for the basal measurements followed by wells with agonists. Otherwise, wash steps are necessary to completely flush out ligands left in the injector tubing.

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