MONITORING THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS (GPCRS) MODULATED BY LIPID OR FREE FATTY ACID AGONISTS

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We have recently demonstrated that the bioluminescent cAMP-Glo™ Assay can track direct activation of adenylate cyclase by forskolin as well as agonists and antagonists that modulate GPCRs by monitoring the level of intracellular cAMP in the cell with receptors that modulate $G\alpha_s$ (Dopamine D1) and those that modulate $G\alpha_i$ (Dopamine D2). Here we extend the application of the cAMP-Glo™ Assay using receptors that are modulated by lipid and free fatty acid agonists. These receptors are of interest since little is known about them and there is conflicting information in the available literature.

Introduction

G protein-coupled receptors (GPCRs) represent one of the largest families of receptors in the human genome. There are almost 800 predicted human GPCRs (1,2), and they respond to a wide variety of extracellular signals such as light, odorants, neurotransmitters and hormones. Nearly half of all prescription drugs target GPCRs (1,2). Since these drugs only target 40–50% of well characterized GPCRs, it is likely that many more GPCRs remain to be explored as drug targets. Thus, there is intense interest in developing novel ligands for orphan receptors, both as potential drugs as well as pharmacological tools to understand cellular physiology. Free fatty acid agonists for GPCRs are of particular interest (3).

The interaction of an activated receptor with a heterotrimeric G protein catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). Subsequent dissociation of the Gα-GTP complex from the Gβγ complex enables both Gα-GTP and Gβγ dimers to interact with a variety of downstream effectors (Figure 1; 4). The alpha subunit is divided into four subgroups: α_s, $\alpha_{i/o}$, α_q and $\alpha_{12/13}$ (4,5). Both $G\alpha_s$ and $G\alpha_{i/o}$ modulate the activity of adenylate cyclase, a key effector enzyme involved in cAMP production. $G\alpha_s$ stimulates adenylate cycles while $G\alpha_{i/o}$ inhibits it. $G\alpha_q$ activates phospholipase C isoform, increasing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol; IP3 increases intracellular calcium concentrations. $G\alpha_{12/13}$ alters the activity of Rho-directed guanine nucleotide exchange factor p115 and GTPase-activating protein RasGAP1.

Several methods are available for studying GPCR modulation: ligand-binding-based assays, measurements of guanine nucleotide exchange, and functional activity-based assays of receptors. Functional activity assays use either reporter-based assays or methods that monitor the intracellular concentrations of the second messengers cAMP or calcium (5–7). Reporter-based assays offer the advantage of wide linearity and sensitivity that allow detection of weak agonists or allosteric modulators (6–8). However, because of the long incubation times required, they are limited by false-positive results, interactions with other signaling pathways and desensitization of receptors. Because of the signal amplification in the pathway, weak agonists can appear as potent agonists (9–11). To address these issues, other functional assays have emerged (5,12,13).

Here we describe using the cAMP-Glo™ Assay, a bioluminescent, homogeneous, and high-throughput assay, for monitoring the modulation of GPCRs that are coupled to $G\alpha_s$ or $G\alpha_i$ proteins, which in turn, modulate adenylate

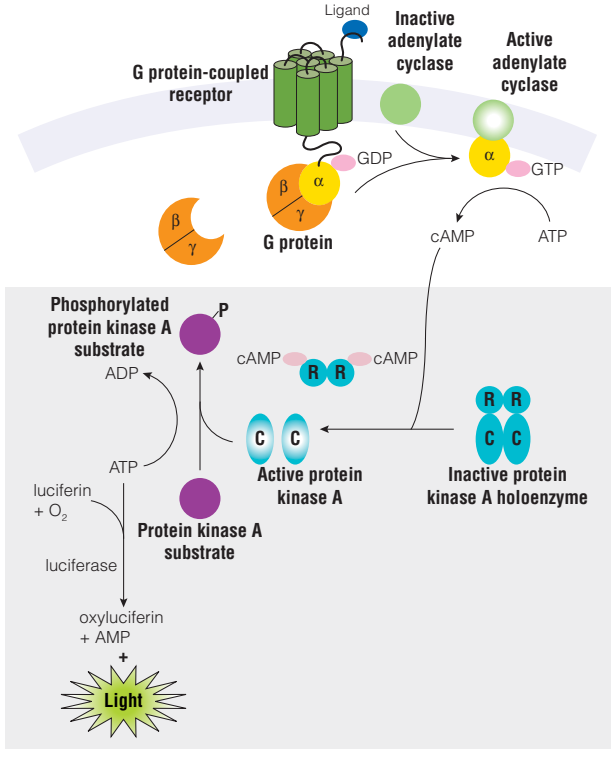

Figure 1. Schematic presentation of cAMP Glo™ Assay. Ligand binding to its cognate G protein-coupled receptor (GPCR) triggers dissociation of the heterotrimeric G (Gαβγ) protein to Gα and Gβγ complex and exchanges GDP for Gα-GTP. The Gα-GTP interacts with adenylate cyclase (AC), resulting in either activation ($G\alpha_s$) or inhibition ($G\alpha_i$) of AC activity. Changes in intracellular cAMP modulate the activity of the tetrameric, inactive cAMP-dependent protein kinase (PKA) into the active free catalytic subunits and the regulatory subunit dimer. The activation of PKA can be monitored by the decrease in ATP substrate in the kinase reaction, and then the remaining ATP can be quantified in a luciferin/luciferase reaction. The amount of light detected is inversely proportional to the intracellular cAMP concentration.

cyclase. The assay is based on cAMP as a potent activator of the tetrameric, inactive cAMP-dependent protein kinase (PKA) resulting in dissociation of its cAMP-bound regulatory subunits and the release of the free, active catalytic subunits (Figure 1). Activation of PKA can be monitored by measuring ATP use in a kinase reaction with a luciferase/luciferin-based reaction. The amount of ATP consumed reflects the activation of PKA by cAMP. The RLU measured indicates the amount of remaining ATP and is reciprocally correlated to both the activity of PKA and the intracellular concentration of cAMP. The assay works with adherent, suspension and frozen cells, offering flexibility and convenience.

Materials and Methods

Stably transfected cells were obtained from Multispan, Inc., Hayward, CA, and were treated and passaged as recommended by the supplier. Three stably transfected cell lines were tested, one signaling through $G\alpha_s$ [HEK293T cells stably transfected with human Prostanoid DP receptor (Multispan Cat.# C1200)], and the other two signaling through $G\alpha_i$ [Chinese hamster ovary cells (CHO) stably transfected with recombinant GPR41 (Multispan Cat.# C1102-1), and HEK293T stably transfected with recombinant GPR43 (Multispan Cat.# C1104)]. The cell lines were used in suspension for all experiments.

Cell Handling and Agonist Treatments

Stably transfected cells were cultured in growth medium until 80% confluent at 5% CO_2 in a 37 °C incubator before harvesting with 0.25% Trypsin-EDTA for 0.5–2 minutes and centrifuging at 1,000 rpm (228 × *g*) for 10 minutes at 25 °C. Cells were resuspended in either serum-free medium or in induction buffer such as Krebs-Ringer Buffer.

For the human Prostanoid DP-transfected cells ($G\alpha_s$-coupled receptors), 10 µl of cells in induction buffer containing 1 mM IBMX (Sigma) and 0.2 mM Ro20-1724 (Sigma) were dispensed into 96-well plates and incubated with 10 µl of 2X the required concentration of forskolin (Sigma) or 2X the required concentration of the agonist [15(R)-PGD2, 15(R)-Prostaglandin D2; Cayman Cat.# 10118] for 20 minutes at room temperature as described in the figure legends. This was followed by adding 20 µl/well of cAMP-Glo™ Lysis Buffer and incubating for 20 minutes at room temperature. Then 40 µl/well of PKA in cAMP-Glo™ Reaction Buffer was dispensed and incubated for an additional 20 minutes. Finally, 80 µl/well of Kinase-Glo® Reagent was added, incubated for 10 minutes at room temperature, and the luminescence was read with a GloMax® 96 Microplate Luminometer (Cat.# E6501).

For GPR41 ($G\alpha_i$-coupled receptors), cells were incubated in 10 µl of induction buffer containing 1 mM IBMX and 0.2 mM Ro20-1724 with 2X the required concentration of forskolin to monitor the effect of forskolin or 10 µl of a mixture of 20 µM forskolin and 2X the required concentration of the agonist sodium propionate to monitor the effect of the agonist on adenylate cyclase inhibition. Forskolin and the forskolin/sodium propionate mixture were incubated for 20 and 25 minutes, respectively.

For GPR43-transfected cells, 10 µl of cells were incubated in induction medium with 1 mM IBMX and 0.2 mM Ro20-1724 but lacking sodium pyruvate before mixing with 10 µl of 2X the required concentration of forskolin to monitor the effect of forskolin on adenylate cyclase activity. To monitor the effect of the agonist sodium acetate, 10 µl of cells were incubated with 4X the required concentration of the agonist sodium acetate (5 µl) and incubated for 10 minutes at room temperature before adding 5 µl of 20 µM forskolin and incubating the cells for an additional 15 minutes. Then 20 µl of cAMP-Glo™ Lysis Buffer was added and incubated for 20 minutes before dispensing 40 µl of cAMP Detection Solution and 80 µl of Kinase-Glo® Reagent into the well as described in the *cAMP-Glo™ Assay Technical Bulletin* #TB357.

In vitro Titration of cAMP and Activation of PKA

Varying concentrations of cAMP (0–1 µM) were added into a mixture of lysis buffer:induction buffer (1:1 v/v), and then mixed with an equal volume of reaction buffer containing PKA and incubated at room temperature for 20 minutes (14). The reaction was terminated by adding an equal volume of Kinase-Glo® Reagent and incubated for an additional 10 minutes. Luminescence was read using the GloMax® 96 Microplate Luminometer, ΔRelative Light Units (ΔRLU) values were plotted against cAMP concentrations, and EC_{50} values were determined using GraphPad Prism® software (14). To establish a cAMP standard curve, we plotted the ΔRLU versus cAMP concentration within the linear range of the assay. The ΔRLU values were calculated by subtracting the RLU at each concentration of cAMP from the RLU value obtained at zero cAMP concentration.

Results and Discussion

The Kinase-Glo® Luminescent Kinase Assay is based on monitoring ATP depletion in a kinase reaction by coupling it to a luciferase/luciferin reaction; activation of PKA by cAMP results in lower ATP levels and decreased luminescence. As shown in Figure 2, the EC_{50} value for cAMP using the Kinase-Glo® Assay is 8.1 nM, a value similar to the literature values for cAMP (3), validating the use of the luminescent kinase assay as a readout platform for activation of PKA by cAMP. We also show that a linear relationship between cAMP concentrations and ΔRLU at each of these concentrations is obtained, and the linearity extends to 60 nM of cAMP (Figure 3).

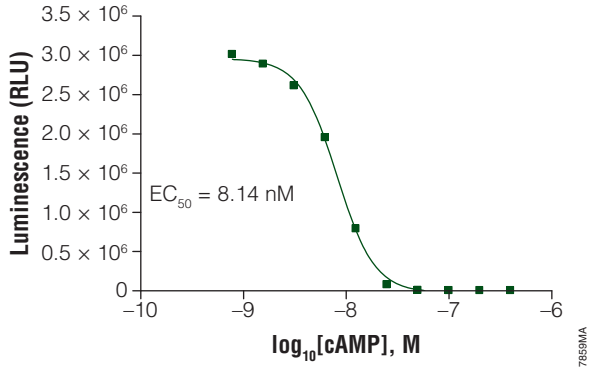

Figure 2. Effect of cAMP concentration on activation of PKA. Increasing cAMP concentration results in dissociation and activation of PKA. The increase in PKA activity is inversely proportional to the amount of relative luminescence units (RLU) as measured by the luciferase/luciferin reaction as described in the Materials and Methods section. The data shown represent means ± standard error of the mean (SEM; n = 3).

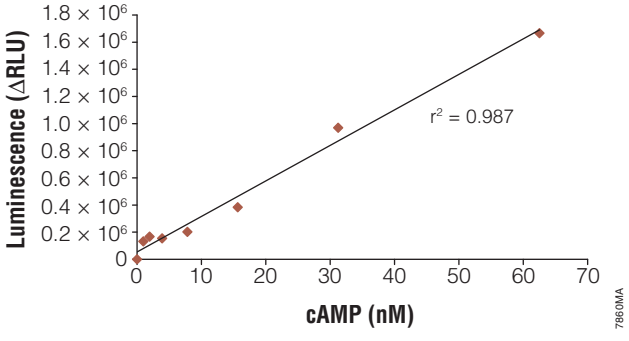

Figure 3. Standard curve showing the linear relationship between cAMP concentration and ΔRLU using the cAMP-Glo™ Assay in 384-well-plate format.

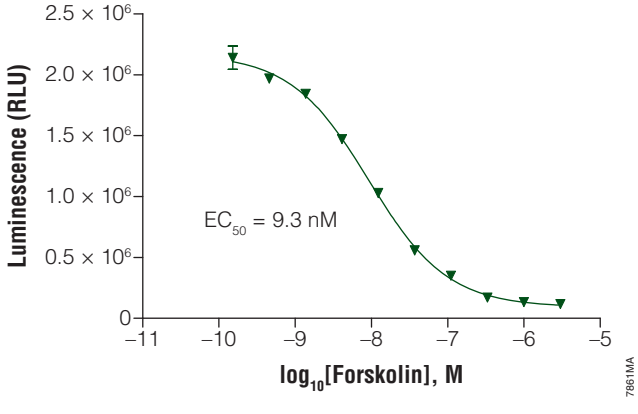

Figure 4. Forskolin titration using human Prostanoid DP-transfected HEK293T cells. DP cells (5,000/well) in a 96-well plate were incubated with various concentrations of forskolin before being processed for cAMP detection as described in the Materials and Methods section. The data shown represent means ± SEM (n = 3).

Forskolin Titration

Since forskolin is a direct activator of adenylate cyclase irrespective of the status of receptors, increasing forskolin concentration results in an increase in intracellular cAMP concentration. Titration of forskolin using DP-transfected HEK 293T cells shows a concentration-dependent increase in intracellular cAMP as a function of forskolin concentration. There was a significant 22-fold change in the RLU values obtained at zero cAMP compared with those obtained at maximum forskolin concentration (EC_{50} for forskolin of 9 nM; Figure 4). Similar profiles were obtained for forskolin titration using GPR41 and GPR43 cells (results not shown).

Agonist Titration

The titration of the agonist 15(R)-prostaglandin D2 in HEK293T cells stably transfected with the human Prostanoid DP receptor showed a concentration-dependent increase in intracellular cAMP concentration with an EC_{50} value of 3.58 nM (Figure 5, Panel A), similar to the reported EC_{50} value for this agonist (6 nM; 15), attesting to the quality of the cAMP-Glo™ Assay.

We also demonstrated the use of the cAMP-Glo™ Assay with $G\alpha_i$-coupled receptors where an agonist response results in a decrease in adenylate cyclase activity. Both GPR41 and GPR43 receptors are activated by free fatty acids within physiological concentration range (16). GPR41 and GPR43 may be coupled to the $G\alpha_{i/o}$ family of proteins (3,17). To test the cAMP-Glo™ Assay with these receptors, we incubated stably transfected cells (GPR41 or GPR43) with 10 µM of forskolin, a concentration found to give maximal adenylate cyclase activation, and variable concentrations of cognate agonists. By increasing the concentration of agonists in the presence of forskolin, we observed the expected corresponding decrease in cAMP and a respective increase in light output. When CHO cells stably transfected with GPR41 were monitored for their intracellular cAMP response to its cognate agonist sodium propionate, a positive relationship between RLU values and propionate concentration was obtained, indicating a decrease in intracellular cAMP with increasing agonist concentration (Figure 5, Panel B). The dose dependency of cAMP on propionate concentrations in the medium is typical of $G\alpha_i$ protein-coupled receptors with an EC_{50} of 90 µM for propionate. Similar studies were done in HEK293T cells stably transfected with GPR43 using 10 µM of forskolin and increasing concentrations of its agonist sodium acetate. The acetate response curve resulted in a decrease in cAMP concentration with an EC_{50} of 885 µM (Figure 5, Panel C). We were able to show that these receptors mediate their response via the $G\alpha_{i/o}$ protein. Furthermore, using fatty acids as agonists, the sensitivity and robustness of the cAMP-Glo™ Assay enabled us to detect changes in intracellular cAMP concentrations for hard-to-demonstrate $G\alpha_{i/o}$ protein-coupled receptors.

Summary

The cAMP-Glo™ Assay can be used to monitor the effect of lipid and fatty acid agonists on GPCR activation by coupling the activation of PKA to a light-generating reaction that is inversely correlated with cAMP levels, a second messenger for receptors. This bioluminescent cAMP assay gives researchers another tool for drug development and exploring cellular physiology.

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Protocol

cAMP-Glo™ Assay Technical Bulletin #TB357
(http://www.promega.com/tbs/tb357/tb357.html)

Ordering Information

Product	Size	Cat.#
cAMP-Glo™ Assay	300 assays	V1501
	3,000 assays	V1502
	30,000 assays	V1503

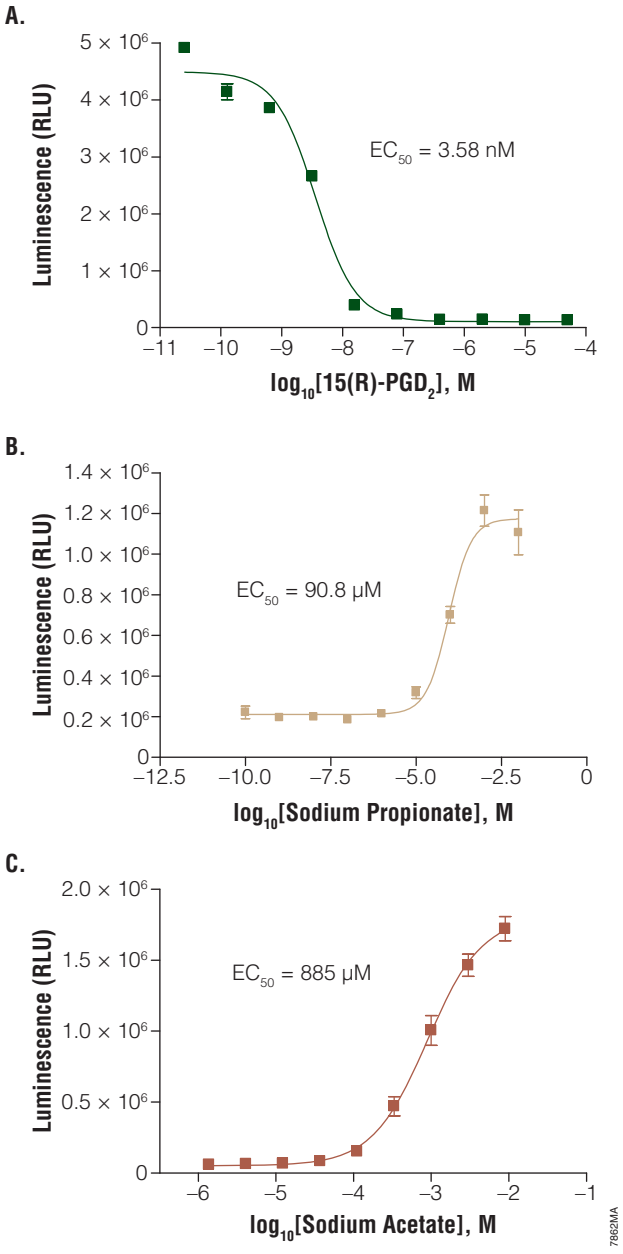

Figure 5. Titration of various receptor agonists. Human Prostanoid DP-transfected HEK293T cells (**Panel A**), GPR41-transfected CHO cells (**Panel B**) and GPR43-transfected HEK293T cells (**Panel C**) were tested as follows: 5,000 DP-transfected cells, a $G\alpha_s$-protein-coupled receptor, were incubated with various concentrations of the agonist 15(R)-PGD2 as described in Materials and Methods. Five thousand GPR41 cells ($G\alpha_i$-coupled receptor) were incubated with 10 µM of forskolin and various concentrations of the agonist sodium propionate to monitor changes in cellular cAMP while 2,500 GPR43 cells ($G\alpha_i$-coupled receptor) were incubated with 10 µM forskolin and various concentrations of the agonist sodium acetate as described in Materials and Methods. The data shown represent means ± SEM (n = 3).