CHAPTER ELEVEN

cAMP Biosensors Applied in Molecular Pharmacological Studies of G Protein-Coupled Receptors

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Contents

Abstract

Cyclic adenosine monophosphate (cAMP) is a common second messenger that mediates numerous biological responses. Intracellular cAMP levels are increased by activation of G_s-coupled G protein-coupled receptors (GPCRs) and decreased by activation of G_i-coupled GPCRs via the adenylyl cyclase. Many end-point assays for quantifying

Methods in Enzymology, Volume 522
ISSN 0076-6879
http://dx.doi.org/10.1016/B978-0-12-407865-9.00011-X
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GPCR-mediated changes in intracellular cAMP levels exist. More recently, fluorescence resonance energy transfer (FRET)-based cAMP biosensors that can quantify intracellular cAMP levels in real time have been developed. These FRET-based cAMP biosensors have been used primarily in single cell FRET microscopy to monitor and visualize changes in cAMP upon GPCR activation. Here, a similar cAMP biosensor with a more efficient mCerulean/mCitrine FRET pair is described for use in the 384-well plate format. After cloning and expression in HEK293 cells, the biosensor is characterized in the 384-well plate format and used for measuring the signaling of the G_s-coupled β_2-adrenergic receptor. The procedures described may be applied for other FRET-based biosensors in terms of characterization and conversion to the 384-well plate format.

1. INTRODUCTION

Cyclic adenosine monophosphate (cAMP) is a common second messenger that is regulated by the activation of G protein-coupled receptors (GPCRs) and mediates numerous biological responses. Quantification of intracellular cAMP levels remains an important methodology in molecular pharmacological studies of GPCRs. More recently, fluorescence resonance energy transfer (FRET)-based cAMP biosensors have been used to monitor real-time GPCR-mediated changes in intracellular cAMP levels primarily by the use of FRET microscopy. In this chapter, a FRET-based cAMP biosensor is described with respect to the generation, characterization, and conversion to a plate reader format.

The second messenger cAMP is formed by the adenylyl cyclases (ACs) by catalysis of ATP to cAMP and inorganic pyrophosphate. Once formed, cAMP can activate protein kinase A (PKA) that in turn phosphorylates intracellular proteins to mediate specific cellular responses. After its formation, cAMP is degraded to AMP by phosphodiesterases. The activity of AC is regulated by GPCRs through the two heterotrimeric GTP-binding protein complexes G_s and G_i. Activation of G_s-coupled receptors lead to increased AC activity and formation of cAMP whereas activation of G_i-coupled receptors lead to inhibition of AC and decreased cAMP levels (Wettschureck & Offermanns, 2005).

Since the identification of cAMP as a second messenger in 1958 (Rall & Sutherland, 1958), methods for detection of cAMP have been developed and improved. Initial techniques relied on separation of accumulated radioactively labeled cAMP from other adenine nucleotides using sequential chromatography. After the development of antibodies for cAMP, various immunoassay strategies were employed, among others, by addition of a

known amount of labeled cAMP to compete with nonlabeled cell-derived cAMP. Today, common technologies for detection of accumulated cAMP include fluorescence polarization and energy transfer assays relying on cAMP antibodies as well as enzyme complementation assays. The mentioned assays are all end-point assays and usually require accumulation of intracellular cAMP to allow detection of cAMP. There are several reviews on detection methods for measuring changes in cAMP induced by GPCRs (Gabriel et al., 2003; Williams, 2004).

In the last few years, biosensors that monitor intracellular cAMP have been developed. The biosensors allow for easy, continuous real-time intracellular cAMP monitoring in contrast to the previously mentioned end-point assays. The different biosensor proteins and their principles of cAMP sensing have been reviewed in detail (Nikolaev & Lohse, 2006). One group of cAMP biosensors belongs to the cyclic nucleotide-gated channels that open and conduct cation influx upon cyclic nucleotide binding (Rich, Tse, Rohan, Schaack, & Karpen, 2001; Strijbos, Pratt, Khan, Charles, & Garthwaite, 1999; Titus et al., 2008). Another group of cAMP biosensors are cAMP-binding proteins fused to fluorescent and/or bioluminescent proteins for FRET and bioluminescence energy resonance transfer (BRET) measurements. The cAMP-binding proteins used are usually based on the cAMP-binding proteins PKA and Epac protein families (Barak et al., 2008; Terrin et al., 2006). Epac is short for "Exchange protein directly activated by cAMP" and these proteins are also known as "cAMP-regulated guanine nucleotide exchange factors". Upon binding of cAMP, the fusion proteins change composition or conformation and thus distance between the fluorescent or bioluminescent proteins, which then give rise to changes in FRET or BRET. The changes in FRET or BRET are very dependent on the conformational change induced in these proteins upon cAMP binding, and optimal positioning of the two fluorescent or bioluminescent proteins in relation to the cAMP-binding domain is crucial for optimal energy transfer. The cAMP biosensors have also been tagged with signal sequences for localization to subcellular compartments, such as the nucleus or membrane, which allow for both temporal and spatial monitoring of cAMP (DiPilato, Cheng, & Zhang, 2004; Terrin et al., 2006).

The FRET-based cAMP biosensors have been used primarily in FRET microscopy of single cells. In this chapter, the generation and use of an optimized FRET-based biosensor consisting of a domain of the cAMP-binding protein Epac1 flanked by cyan and yellow fluorescent protein on either side in a plate reader format are described. The approach described here can, in

principle, be applied to the development of other biosensors of interest for studying GPCR signaling. For example, a number of microscopy FRET-based biosensors have been described for IP_3 (Matsu-ura et al., 2006; Tanimura et al., 2009) and activation of PKC and ERK (Harvey et al., 2008; Sato, Kawai, & Umezawa, 2007; Violin, Zhang, Tsien, & Newton, 2003). To convert such biosensors into a plate reader format, the procedures described for the cAMP biosensor below may be followed.

2. GENERATION OF AN EPAC1-BASED CAMP BIOSENSOR CELL LINE WITH AN OPTIMIZED FLUORESCENT PROTEIN FRET PAIR

2.1. Generation of DNA encoding the optimized FRET cAMP biosensor

Based on the literature for cAMP biosensors (referenced above), a full-length cAMP-binding protein Epac1 appears attractive for a plate-based cAMP biosensor assay because of the reported large FRET ratio shifts upon binding of cAMP. In principle, the biosensor is similar to an EPac1 cAMP biosensor described previously (Violin et al., 2008) except that a more efficient fluorescent protein FRET pair is used. The mCerulean/mCitrine (mCer/mCit) FRET pair has been described as an efficient FRET pair (Rizzo, Springer, Granada, & Piston, 2004) and is used to flank the cAMP-binding Epac1 domain. Initially, an engineered expression vector for FRET biosensors is generated. After the CMV promoter in a mammalian expression vector, DNA that codes for the following is inserted: (1) mCer, (2) a small multiple cloning sequence (MCS) in frame with (3) mCit. The cDNA encoding the Epac1 amino acids 149–881 is then amplified and subcloned in between the two fluorescent proteins using the introduced multiple cloning sequence.

Materials

- Epac1 DNA fragment
  - cDNA encoding the human Epac1 protein (IMAGE-clone no. 4414360, NCBI accession number BC017728)
  - sense primer Epac1 consisting of four nonsense bases (italics), *Kpn*I restriction site (bold), and bases coding for amino acid 149–155 of Epac1: 5′-*GAAT***GGTACC**CCCGTGGGAACTCATGAGATG-3′
  - antisense primer Epac1 consisting of four nonsense bases (italics), *Eco*RI restriction site (bold), and bases coding for amino acid 881–875 of Epac1: 5′-*GTAT***GAATTC**TGGCTCCAGCTCTCGGGAGAG-3′

- Monomeric Cerulean fluorescent protein DNA
  - cDNA encoding mCer (obtained by introducing the following mutations in ECFP: S72A/Y145A/H148D (Rizzo et al., 2004) and A206K (monomeric))
  - sense primer mCer consisting of four nonsense bases (italics), an *Nhe*I restriction site (bold), a kozak sequence (underlined), and bases coding for the initial amino acids of ECFP/mCer: 5′-*GTAT***GCTAGC**GCCACCATGGTGAGCAAGGGCGAGGAG-3′
  - antisense primer mCer consisting of four nonsense bases (italics), a *Kpn*I restriction site (bold), and bases coding for the last amino acids of ECFP/mCer without a stop codon: 5′-*GAAT***GGTACC**AGCCTTGTACAGCTCGTCCATGCCG-3′
- Monomeric Citrine fluorescent protein DNA
  - cDNA encoding mCit (obtained by introducing the following mutations in EYFP: Q69M (Griesbeck, Baird, Campbell, Zacharias, & Tsien, 2001) and A206K (monomeric))
  - sense primer mCit consisting of four nonsense bases (italics), an *Eco*RI restriction site (bold), and bases coding for the initial amino acids of EYFP/mCit: 5′-*GTAA***GAATTC**ATGGTGAGCAAGGGCGAGGAG-3′
  - antisense primer mCit consisting of four nonsense bases (italics), an *Xba*I restriction site (bold), a stop codon (underlined), and bases coding for last amino acids of EYFP/mCit: 5′-*GTAT***TCTAGA**TTACTTGTACAGCTCGTCCATGCC-3′
- pcDNA3.1(+)zeo expression vector (Invitrogen)
- Standard molecular biology reagents, including restriction enzymes *Nhe*I, *Kpn*I, *Eco*RI, and *Xba*I.

The engineered expression vector for FRET biosensors (pcDNA3.1(+)zeo-mCer-MCS-mCit vector) is generated by sequential subcloning of PCR-amplified DNA encoding the mCer and mCit into the MCS of pcDNA3.1(+)zeo. mCer DNA for subcloning is amplified by the above-mentioned primers that incorporate a 5′-end *Nhe*I and a 3′-end in-frame *Kpn*I restriction site. mCit DNA is amplified by primers that incorporate 5′-end in-frame *Eco*RI and 3′-end *Xba*I restriction sites. The resultant engineered expression vector (pcDNA3.1(+)zeo-mCer-MCS(*Kpn*I–*Sac*I–*Bam*HI–*Spe*I–*Eco*RI)-mCit) may be used for other FRET biosensors than the Epac1-based cAMP biosensor described here. To generate the Epac1 cAMP biosensor, the Epac1 cDNA, which can be obtained as an IMAGE-clone, encoding the amino acids 149–881 is amplified by PCR using primers that incorporate in-frame

5′-end *Kpn*I and 3′-end *Eco*RI restriction sites. The Epac1 PCR product is then subcloned into the pcDNA3.1(+)zeo-mCer-MCS-mCit vector, and the resultant cAMP biosensor, hereafter referred to as Epac149 biosensor, is sequence verified.

2.2. Stable expression of the cAMP biosensor in HEK293 cells

In order to test the Epac149 biosensor responsiveness to cAMP, the biosensor construct is first stably expressed in HEK293 cells to ensure a sufficient signal from all cells. Depending on the sensitivity of the detection apparatus, it may not be necessary to stably express the biosensor to test its responsiveness to cAMP. When carried out on the Envision plate reader from Perkin Elmer (see below), it is possible to transiently transfect the biosensor and still obtain high fluorescence intensities of mCer and mCit for determination of the FRET ratio.

Materials

- Standard cell culture plasticware, medium, serum, etc.
- Transfection reagent of choice
- HEK293 cell line (ATCC CRL-1573)
- Zeocin (Invitrogen)
- A DNA preparation of the above-mentioned expression vector pcDNA3.1 (+)zeo containing the Epac149 biosensor

Apparatus

- Fluorescence microscope with filters for CFP and/or YFP fluorescence. A microscope with GFP filters will likely also work.

The stable expressing cell line is established by limited dilution cloning. HEK293 cells are transfected with the Epac149 biosensor by the calcium phosphate method (or any other available transfection method). Two days after transfection, the cells are passaged and diluted into different densities (limited dilution cloning) and selected with 50 μg/mL zeocin. A typical dilution range is the originally transfected 100 mm tissue culture dish diluted into separate plates in dilutions of 1:5, 1:25, 1:125, 1:500, and 1:1000. After 2 weeks of selection with change of medium every third day, single colonies can typically be picked from one or two of the diluted plates. Using a fluorescence microscope, colonies that express the biosensor at low, medium, and high levels (based on apparent visual fluorescence) are picked and expanded. The picked colonies are expanded first in the 6-well plates and then in 3 mm × 100 mm tissue culture dishes for subsequent tests for responsiveness to increase intracellular cAMP (one dish) and for freezing of cell aliquots (two dishes).

3. cAMP BIOSENSOR CHARACTERIZATION

3.1. Biosensor responsiveness for increases in intracellular cAMP and emission spectrum characterization

As the biosensor cell line will be used to quantify the functional responses of G_s-coupled GPCRs, it is important to assess whether the intracellular cAMP levels produced by GPCR signaling are within the dynamic range of the biosensor. Also, by collecting emission spectra of mCer and mCit, it is possible to assess the extent of conformational change upon cAMP binding. To test Epac149 biosensor expressing cells for responsiveness to intracellular cAMP, changes in emission spectra of mCer and mCit were recorded under conditions of buffer, forskolin, and isoproterenol (ISO) stimulation. Forskolin directly activates the AC and will induce a large increase in intracellular cAMP. ISO is an agonist of β-adrenergic receptors of which the G_s-coupled β_2-adrenergic receptor (β_2-AR) is endogenously expressed in native HEK293 cells.

Apparatus

- Swinging-bucket cell centrifuge
- Spectramax-3 spectrofluorimeter (Horiba Scientific) or similar spectrofluorimeter that will record the emission spectrum of the biosensor

Materials

- DPBS buffer: DPBS (without Mg^{2+} and Ca^{2+})
- DPBS–EDTA buffer: DPBS (without Mg^{2+} and Ca^{2+}) with 2 m*M* EDTA
- HBSS buffer: HBSS (without Mg^{2+} and Ca^{2+}) supplemented with 20 m*M* HEPES pH 7.5, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$
- HBSS cell suspension buffer: HBSS buffer supplemented with 0.2% bovine serum albumin (BSA)
- Forskolin and isoproterenol (Sigma)

Bring adherent biosensor-expressing cells from a near-confluent 100-mm tissue culture dish into suspension by removing the growth medium, rinsing the dish once with 5 mL DPBS buffer, and incubating the dish with 5 mL DPBS–EDTA buffer for up to 5 min. After the cells have detached, titrate up and down to rinse off the remainder of the cells and bring the cells into single cell suspension. Spin down at 1000 rpm for 5 min in a swinging-bucket centrifuge. Carefully remove the supernatant and resuspend the cell pellet in 1 mL HBSS cell suspension buffer. Count the cells and dilute to 1.2×10^6 cells/mL

in HBSS cell suspension buffer. The cell suspension may be left in the dark at room temperature while preparing the compound solutions.

Then prepare 20 μ*M* ISO and 20 μ*M* forskolin solutions in HBSS buffer.

For measurements in the spectrofluorimeter, mix in a cuvette 100 μL cell suspension and 100 μL HBSS buffer with 20 μ*M* forskolin or 20 μ*M* ISO to give final concentrations of 10 μ*M*. Then place the cuvette in the spectrofluorimeter, and collect emission spectra of mCer and mCit every 5 min as the kinetics of intracellular cAMP may change over time. For the Spectramax-3, the cell suspension is excited at 434 nm with a slit of 5 nm, and emission spectrum collected from 450 to 570 nm with a slit of 5 nm. Other sensitive spectrofluorimeters with monochromators should also be able to record the emission spectrum of the biosensor. One alternative is the Tecan Saphire[2].

The biosensor spectra typically look like the spectra in Fig. 11.1A, which are recorded after 20 min. As shown in Fig. 11.1A, there is a clear decrease in the emission of mCit and a tendency of an increase in mCer emission upon forskolin and ISO stimulation compared to the buffer level. The decrease in FRET ratio suggests that mCer and mCit move apart upon an increase in the intracellular cAMP formation (Fig. 11.1B). The FRET ratio change observed by ISO

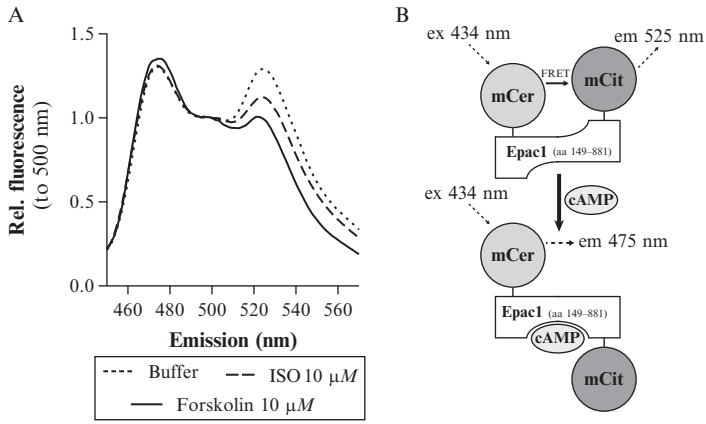

Figure 11.1 Emission scan of the Epac149 biosensor (A) and conceptual drawing of the Epac149 biosensor conformational changes upon cAMP binding (B). (A) The emission (em) spectrum of cells expressing the Epac149 biosensor was collected from 450 to 570 nm after excitation (ex) at 434 nm. Stimulation with isoproterenol (ISO) and forskolin resulted in a clear decrease in the mCitrine emission peak at 525 nm. (B) Upon binding of cAMP to the Epac149 biosensor, the distance between the two fluorescent proteins of the FRET pair increases, resulting in less FRET. The Epac149 biosensor consists of the amino acids 149–881 of the Epac1 cAMP-binding protein flanked by mCerulean and mCitrine on either side.

compared to buffer levels is important as this is the window that may be expected from activation of a G_s-coupled GPCR. Having ensured that changes in FRET occur upon stimulation with compounds that increase the intracellular cAMP concentration, the dynamic range and relation between changes in FRET ratio and cAMP concentrations may now be established.

3.2. Correlation of FRET ratio with intracellular cAMP concentrations in a plate reader format

After identification of a responsive cell line that give a clear shift in FRET ratio upon forskolin and ISO, the dynamic range of the cAMP biosensor given by the EC_{50} of cAMP and the slope of the curve is determined in the plate reader format. For estimation of the dynamic range, known concentrations of cAMP are added to permeabilized cells in a buffer corresponding to the intracellular fluid (ICF), and the change in FRET ratio is measured. From a graph depicting the FRET ratio as a function of cAMP concentration, it is now possible to perform a nonlinear regression fit and calculate the intracellular concentration of cAMP from the FRET ratio observed.

Apparatus

- Envision plate reader equipped with the following filters and dichroic mirror
  - Excitation filter for mCer: CFP 430 (barcode 138), X430 CWL = 430 nm, BW = 24 nm, $T_{min} = 90\%$
  - Emission filter for mCer: CFP 470 (barcode 240), M470 CWL = 470 nm, BW = 24 nm, $T_{min} = 90\%$
  - Emission filter for mCit: YFP 535 (barcode 274), M535 CWL = 535 nm, BW = 30 nm, $T_{min} = 90\%$
  - Dichroic mirror: CFP/YFP (barcode 428), D450_515

Materials

- DPBS buffer: DPBS (without Mg^{2+} and Ca^{2+})
- DPBS–EDTA buffer: DPBS (without Mg^{2+} and Ca^{2+}) with 2 m*M* EDTA
- ICF buffer: 10 m*M* HEPES-KOH buffer (pH 7.4) with 20 m*M* NaCl, 120 m*M* KCl, 1 m*M* EGTA, 330 μ*M* $CaCl_2$, and 0.1% saponin
- ICF cell suspension buffer: ICF buffer supplemented with 0.2% BSA. (Make a 10% BSA stock, filter sterilize, and store in a refrigerator for up to 1 month.)
- 1% Saponin (Sigma) solution in ICF buffer
- cAMP (Sigma)
- Black 384-well plate, nontreated and with a black bottom (e.g., Corning 384 plate, cat. no. 3575)

First make serial dilutions of cAMP in ICF buffer in 2× the final concentration. The final concentrations used in Fig. 11.2 are 5 m*M*, 1 m*M*, 100 μ*M*, 10 μ*M*, 1 μ*M*, and 0.1 μ*M*. Plate the ICF buffer with different cAMP concentrations in 20 μL volumes in duplicate in a 384-well plate. Then detach cells (in a near-confluent 100-mm tissue culture dish) and bring into single cell suspension as described in Section 3.1. After pelleting and careful removal of the supernatant, resuspend cells in 0.9 mL ICF cell suspension buffer and count the cells. Add 100 μL of a 1% saponin solution in ICF to a final concentration of 0.1% and leave for 5 min at room temperature to permeabilize cells. Then pellet cells in a swinging-bucket centrifuge and resuspend in ICF cell suspension buffer to 1.2×10^6 cells/mL. Prepare the plate reader for measuring the fluorescence of mCer and mCit upon mCer excitation continuously. Transfer 20 μL of cell suspension to the wells containing buffer with different concentrations of cAMP. Initiate reading of the plate immediately as cAMP upon contact with cells is likely to be degraded because of phosphodiesterases. If this is the case it should be evident by depicting the time course of changes in the FRET ratio, where the maximal FRET ratio change by exogenous cAMP normally is observed on the first or second cycle read. Determine the time point that had the highest FRET change for the exogenous cAMP and plot against the cAMP

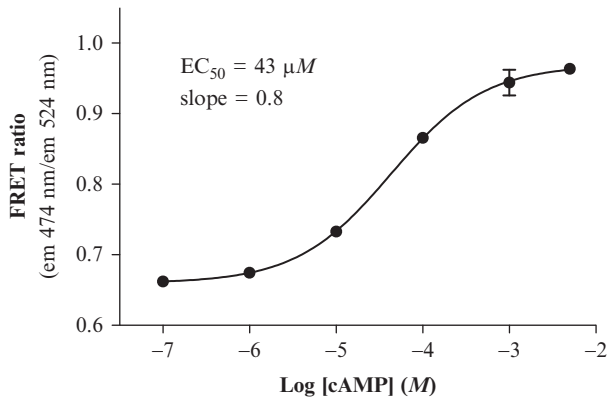

Figure 11.2 Relationship between the cAMP concentration and the measured FRET ratio. Varying concentrations of cAMP in intracellular-like fluid were incubated with permeabilized Epac149 biosensor-expressing cells and resulted in a change in the FRET ratio. The cAMP/FRET ratio relationship can be fitted to a sigmoidal concentration–response curve by nonlinear regression, which allows for calculation of the intracellular cAMP concentration. In principle, the FRET ratio decreases upon increasing cAMP concentrations, but here, the FRET ratio is calculated as the ratio between the fluorescence of mCerulean and mCitrine to obtain a graph that increases upon increases in cAMP concentrations.

concentration to establish the relationship between cAMP and the FRET ratio (Fig. 11.2). The EC_{50} and slope may be determined for the biosensor by nonlinear regression fitting using the program GraphPad Prism 5.0 d.

After establishing the cAMP/FRET ratio relationship in the plate reader format, the Epac149 biosensor cell line can be used to determine the intracellular cAMP concentration.

4. ASSAY FOR STUDYING GPCR SIGNALING AS EXEMPLIFIED BY G_S-COUPLED β_2-ADRENERGIC RECEPTOR ACTIVATION

The cAMP biosensor has the clear advantage of continuous real-time monitoring of intracellular cAMP in contrast to end-point cAMP assays. The number of assay points and throughput can also be increased significantly in the 384-well plate format as compared to the FRET-based microscopy format. In the following, a general protocol for assaying the cAMP kinetics of a G_s-coupled GPCR and determination of ligand potency is described. The β_2-AR, which is endogenously expressed in the HEK293 cell line, will be used as a representative G_s-coupled receptor. Besides the kinetic profile of β_2-AR-mediated cAMP signaling by ISO, the inactivation of receptor signaling by the inverse agonist ICI-118,551 (ICI) is also determined.

Materials

- DPBS buffer: DPBS (without Mg^{2+} and Ca^{2+})
- DPBS–EDTA buffer: DPBS (without Mg^{2+} and Ca^{2+}) with 2 m*M* EDTA
- HBSS buffer: HBSS (without Mg^{2+} and Ca^{2+}) supplemented with 20 m*M* HEPES, pH 7.5, 1 m*M* $CaCl_2$, and 1 m*M* $MgCl_2$
- HBSS cell suspension buffer: HBSS buffer supplemented with 0.2% BSA
- Forskolin (Sigma)
- β_2-AR ligands ISO (agonist) and ICI (inverse agonist)

Apparatus

- Envision equipped with the filters and dichroic mirror described in Section 3.2.

Make the ligand dilutions of ISO, ICI, and forskolin in 2× the final concentration. The final concentration range for ISO is from 100 μ*M* to 1 p*M* in 10-fold dilutions. Typically, duplicate 20 μL aliquots of the different concentrations of ISO are added to separate wells of a 384-well plate. In this experiment, three duplicates of each concentration are made. ICI and forskolin are used at 10 μ*M* and are also added to separate wells in duplicate.

Detach cells and isolate the cell pellet as described in Sections 3.1 and 3.2 with DPBS and DPBS–EDTA. Resuspend the cell pellet in HBSS cell suspension buffer and leave in the dark at room temperature until use. The actual time from cell detachment to performance of measurement does not seem to be crucial. Usually, the cells are left in the final dilution suspension for 30 min before they are added to the 384-well plate. Measurements on cells may be performed up to at least 5 h after dislodging and resuspension.

Prepare the Envision plate reader. In this example, the protocol is set up so that each well can be read every 1 min for 140 min, but this can obviously be modified. As in the previous section, fluorescence of mCer and mCit is measured after excitation of mCer.

Add cells to all the wells and start the repeated reading of the plate as soon as possible to obtain the initial kinetics of cAMP generation (see below for options on how to obtain the initial kinetics immediately after receptor activation). After 5 min, the plate reader is paused and 10 μM ICI is added to the wells containing one of the ISO concentration–response curves (again refer below for options how to add ICI without pausing the read). Then reading is resumed and after 30 min, ICI is added to another set of ISO concentration–response curves. The reading is resumed again and finally stopped after a total of about 140 min.

After transfer of data and analysis in GraphPad Prism, data typically look like Fig. 11.3. It is evident that various concentrations of ISO elicit different cAMP kinetic profiles that return toward the basal level over time (Fig. 11.3A). This is consistent with receptor desensitization in the continued presence of agonist. Concentration–response curves may be made at different time points. Typically, the time point for determining the maximal cAMP response by various concentrations of agonist is chosen around the time where the highest basal to maximal response is obtained, in this case in the interval between 8 and 15 min. Plotting the FRET ratio as a function of the agonist concentration generates the typical concentration–response curve of a particular ligand, in this case ISO (Fig. 11.3B). Addition of the inverse agonist ICI at 5 and 30 min suggests that the cAMP signaling by ISO can be immediately inhibited by a competing ligand (Fig. 11.3C). By the use of the previously established relationship between the cAMP concentration and the FRET ratio (Fig. 11.2), the actual intracellular cAMP concentration induced by the ligands may be determined (Fig. 11.3D).

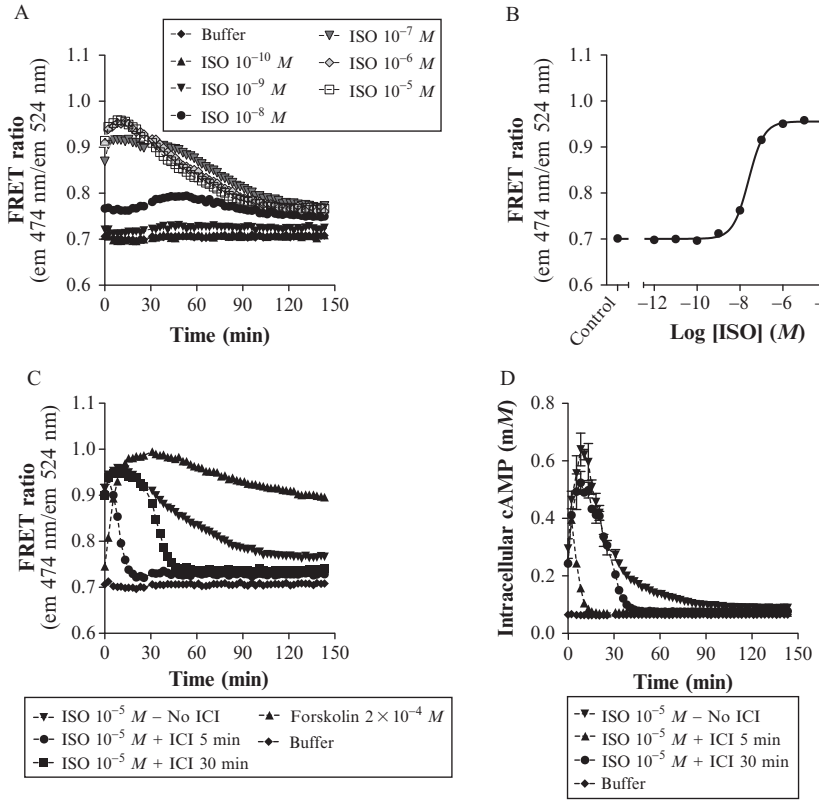

Figure 11.3 Isoproterenol response kinetics in Epac149 biosensor-expressing HEK293 cells. HEK293 cells endogenously express the β_2-adrenergic receptor that was used as a representative G_s-coupled receptor. (A) Varying concentrations of isoproterenol (ISO) elicit different cAMP kinetic profiles. (B) Concentration–response curve of ISO obtained by plotting the average FRET ratio in the interval 8–15 min against different concentrations of ISO. (C) ISO and forskolin response kinetics and complete inhibition of ISO-induced cAMP formation by the inverse agonist ICI-118,551 when added at 5 and 30 min. (D) Conversion of ISO response kinetics (Fig. 11.3C) with and without ICI-118,551 addition at 5 and 30 min to intracellular cAMP concentrations. The intracellular cAMP concentration is determined by the use of the established relationship between the cAMP concentration and the FRET by extrapolation of the nonlinear regression. For Fig. 11.3A–C, the FRET ratio, in principle, decreases upon increasing cAMP concentrations, but here, the FRET ratio is calculated as the ratio between the fluorescence of mCerulean and mCitrine to obtain a graph that increases upon increases in cAMP concentrations.

5. MODIFICATIONS TO THE GENERAL cAMP KINETICS ASSAY

In the development and use of the Epac149 biosensor and the Epac149 biosensor HEK293 cell line, a number of observations are worth mentioning in relation to the further use of the Epac149 biosensor for quantifying G_s-coupled GPCR cAMP signaling.

5.1. Addition of cells and ligand using the Envision pumps

In the development of the Epac149 biosensor assay, experiments with the two integrated pumps on the Envision plate reader have also been performed. The Envision plate reader pump may add both cells and a ligand solution to selected wells. Performing the above-mentioned studies where ICI is added to specific wells at certain time points may thus be automated by the use of the Envision pump. By automating the addition, it will not be necessary to pause the instrument and add the ligand manually, and continuous measurements may be performed.

Similarly, the pump can be used to add cells to specific wells. Thus, in one setup, the ligands at different concentrations can first be added manually to different wells. Then the plate with ligands can be loaded into the plate reader and cells in suspension added by the pump. Using this approach, the initial kinetics of changes in cAMP by activation of GPCRs can be obtained, in contrast to the earlier described approach of adding cells manually.

5.2. Cell density independence of FRET ratio and use of fluorescence as a measure of cell number

For Epac149 biosensor measurement of cAMP, 20,000–30,000 cells per 384-well are usually used. When measuring mCer and mCit fluorescence, this amount consistently corresponds to fluorescence intensity counts of around 200,000 and 300,000. Thus, instead of counting cells after dislodging, the cell suspension fluorescence is measured initially and then adjusted to 200,000–300,000 counts by dilution in cell suspension buffer.

In a number of cases, the FRET ratio has been measured at cell densities other than 20,000–30,000 cells per 384-well. Although the absolute number of cells is different from well to well (as measured by the absolute fluorescence intensities), the FRET ratio is consistently the same. In fact, fluorescence can vary from 100,000 to 1,500,000 counts without the FRET ratio being

different. Outside this interval, however, the FRET ratio under the same conditions tends to be significantly different.

5.3. Overexpression of GPCRs and effect of addition of phosphodiesterase inhibitors

In an attempt to enhance the signaling of the β_2-AR, the stable expressing Epac149 cell line has been stably overexpressed with the β_2-AR. The cAMP kinetics upon activation of the overexpressing β_2-AR cell line is very different from the parent Epac149 stably expressing cell line where the β_2-AR is expressed endogenously at lower levels (Fig. 11.4). It is evident that the cAMP concentration for the overexpressing cell line does not return to basal levels over time as seen for the low β_2-AR expressing parent cell line. This is likely due to the presence of spare receptors and the inability of the intracellular machinery for desensitization of GPCRs to handle the gross overexpression of the β_2-AR. Clearly, the lack of desensitization in the overexpressing cell line is advantageous in a ligand-screening setting but is likely far from the physiological response.

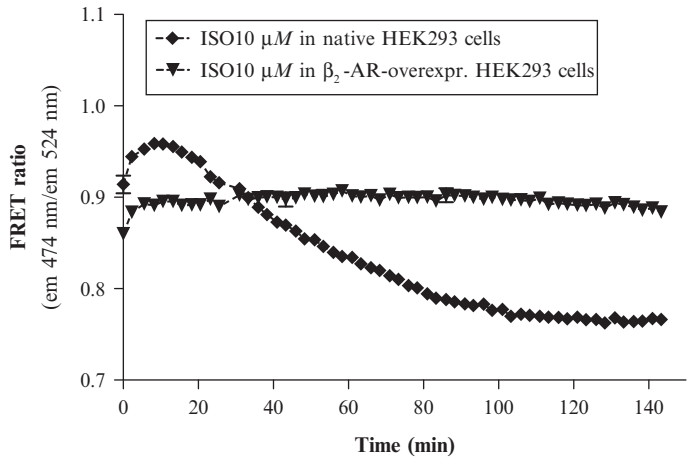

Figure 11.4 Isoproterenol response kinetics in native and β_2-adrenergic receptor (β_2-AR)-overexpressing HEK293 cells. There is a clear difference in the isoproterenol (ISO) kinetics between the two cell lines as the ISO response does not return to basal levels over time for the overexpressing cell line. Depending on the purpose of the experiments performed, this may or may not be advantageous. In principle, the FRET ratio decreases upon increasing cAMP concentrations, but here, the FRET ratio is calculated as the ratio between the fluorescence of mCerulean and mCitrine to obtain a graph that increases upon increases in cAMP concentrations.

A similar prolongation of GPCR signaling may be obtained by the addition of phosphodiesterase inhibitors such as 3-isobutyl-1-methylxanthine (IBMX). The addition of IBMX also allows the detection of the basal activity of GPCRs and characterization of inverse agonists. For example, a slow increase in intracellular cAMP concentrations in the β_2-AR overexpressing cell line has been observed in the presence of IBMX as compared to cells in the presence of the inverse agonist ICI and IBMX (data not shown).

5.4. Transient cotransfection of Epac149 biosensor and GPCRs

The Epac149 biosensor has also been used to follow the signaling kinetics of G_s-coupled GPCRs in a transient cotransfection setting. β_2-AR receptor mutants have successfully been cotransfected with the Epac149 biosensor in BHK cells, which do not endogenously express the β_2-AR. In these experiments, the ISO EC_{50} and basal activity for the mutants have been established (data not shown). Other G_s-coupled GPCRs have also been transiently expressed in the stably expressing Epac149 cell line or transiently cotransfected with the Epac149 biosensor to assess the cAMP kinetics of different agonists (data not shown).

ACKNOWLEDGMENTS

We thank Professor Brian Kobilka for use of the Spectramax-3 spectrofluorimeter. This work was supported by The Lundbeck Foundation (J. M. M. and H. B. -O.) and The Danish Council for Independent Research—Medical Sciences (J. M. M. and H. B. -O.).

REFERENCES

Barak, L. S., Salahpour, A., Zhang, X., Masri, B., Sotnikova, T. D., Ramsey, A. J., et al. (2008). Pharmacological characterization of membrane-expressed human trace amine-associated receptor 1 (TAAR1) by a bioluminescence resonance energy transfer cAMP biosensor. *Molecular Pharmacology*, *74*, 585–594.

DiPilato, L. M., Cheng, X., & Zhang, J. (2004). Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 16513–16518.

Gabriel, D., Vernier, M., Pfeifer, M. J., Dasen, B., Tenaillon, L., & Bouhelal, R. (2003). High throughput screening technologies for direct cyclic AMP measurement. *Assay and Drug Development Technologies*, *1*, 291–303.

Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A., & Tsien, R. Y. (2001). Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *The Journal of Biological Chemistry*, *276*, 29188–29194.

Harvey, C. D., Ehrhardt, A. G., Cellurale, C., Zhong, H., Yasuda, R., Davis, R. J., et al. (2008). A genetically encoded fluorescent sensor of ERK activity. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 19264–19269.

Matsu-ura, T., Michikawa, T., Inoue, T., Miyawaki, A., Yoshida, M., & Mikoshiba, K. (2006). Cytosolic inositol 1,4,5-trisphosphate dynamics during intracellular calcium oscillations in living cells. *The Journal of Cell Biology*, *173*, 755–765.

Nikolaev, V. O., & Lohse, M. J. (2006). Monitoring of cAMP synthesis and degradation in living cells. *Physiology*, *21*, 86–92.

Rall, T. W., & Sutherland, E. W. (1958). Formation of a cyclic adenine ribonucleotide by tissue particles. *The Journal of Biological Chemistry*, *232*, 1065–1076.

Rich, T. C., Tse, T. E., Rohan, J. G., Schaack, J., & Karpen, J. W. (2001). In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. *The Journal of General Physiology*, *118*, 63–78.

Rizzo, M. A., Springer, G. H., Granada, B., & Piston, D. W. (2004). An improved cyan fluorescent protein variant useful for FRET. *Nature Biotechnology*, *22*, 445–449.

Sato, M., Kawai, Y., & Umezawa, Y. (2007). Genetically encoded fluorescent indicators to visualize protein phosphorylation by extracellular signal-regulated kinase in single living cells. *Analytical Chemistry*, *79*, 2570–2575.

Strijbos, P. J., Pratt, G. D., Khan, S., Charles, I. G., & Garthwaite, J. (1999). Molecular characterization and in situ localization of a full-length cyclic nucleotide-gated channel in rat brain. *The European Journal of Neuroscience*, *11*, 4463–4467.

Tanimura, A., Morita, T., Nezu, A., Shitara, A., Hashimoto, N., & Tojyo, Y. (2009). Use of fluorescence resonance energy transfer-based biosensors for the quantitative analysis of inositol 1,4,5-trisphosphate dynamics in calcium oscillations. *The Journal of Biological Chemistry*, *284*, 8910–8917.

Terrin, A., Di Benedetto, G., Pertegato, V., Cheung, Y. F., Baillie, G., Lynch, M. J., et al. (2006). PGE(1) stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: Role of compartmentalized phosphodiesterases. *The Journal of Cell Biology*, *175*, 441–451.

Titus, S., Neumann, S., Zheng, W., Southall, N., Michael, S., Klumpp, C., et al. (2008). Quantitative high-throughput screening using a live-cell cAMP assay identifies small-molecule agonists of the TSH receptor. *Journal of Biomolecular Screening*, *13*, 120–127.

Violin, J. D., DiPilato, L. M., Yildirim, N., Elston, T. C., Zhang, J., & Lefkowitz, R. J. (2008). Beta2-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *The Journal of Biological Chemistry*, *283*, 2949–2961.

Violin, J. D., Zhang, J., Tsien, R. Y., & Newton, A. C. (2003). A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *The Journal of Cell Biology*, *161*, 899–909.

Wettschureck, N., & Offermanns, S. (2005). Mammalian G proteins and their cell type specific functions. *Physiological Reviews*, *85*, 1159–1204.

Williams, C. (2004). cAMP detection methods in HTS: Selecting the best from the rest. *Nature Reviews. Drug Discovery*, *3*, 125–135.