

A cAMP Biosensor-Based High-Throughput Screening Assay for Identification of Gs-Coupled GPCR Ligands and Phosphodiesterase Inhibitors

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Abstract

Cyclic adenosine 3',5'-monophosphate (cAMP) is an important second messenger, and quantification of intracellular cAMP levels is essential in studies of G protein-coupled receptors (GPCRs). The intracellular cAMP levels are regulated by the adenylyl cyclase (AC) upon activation of either Gs- or Gi-coupled GPCRs, which leads to increased or decreased cAMP levels, respectively. Here we describe a real-time Förster resonance energy transfer (FRET)-based cAMP high-throughput screening (HTS) assay for identification and characterization of Gs-coupled GPCR ligands and phosphodiesterase (PDE) inhibitors in living cells. We used the β_2 -adrenergic receptor (β_2 AR) as a representative Gs-coupled receptor and characterized two cell lines with different expression levels. Low receptor expression allowed detection of desensitization kinetics and delineation of partial agonism, whereas high receptor expression resulted in prolonged signaling and enabled detection of weak partial agonists and/or ligands with low potency, which is highly advantageous in large HTS settings and hit identification. In addition, the assay enabled detection of β_2 AR inverse agonists and PDE inhibitors. High signal-to-noise ratios were also observed for the other representative Gs-coupled GPCRs tested, GLP-1R and GlucagonR. The FRET-based cAMP biosensor assay is robust, reproducible, and inexpensive with good Z factors and is highly applicable for HTS.

Keywords

high-throughput screening (HTS), cAMP biosensor, FRET, G protein-coupled receptor (GPCR), Gs-coupled receptor activation

Introduction

Cyclic adenosine 3',5'-monophosphate (cAMP) is a common second messenger that serves to propagate intracellular signal transduction. Formation of cAMP is regulated via adenylyl cyclases (ACs), which upon activation catalyze adenosine triphosphate (ATP) into cAMP and inorganic pyrophosphate. cAMP can lead to activation of protein kinase A (PKA) that regulates the activity of several other cellular proteins to mediate numerous biological responses. Once formed, cAMP is degraded to adenosine 5'-monophosphate (AMP) by specific phosphodiesterases (PDEs). G protein-coupled receptors (GPCRs) modulate intracellular cAMP levels through the heterotrimeric GTP binding protein complexes Gs and Gi. Activation of Gs-coupled GPCRs and thus G α_s results in stimulation of the AC and formation of cAMP, whereas activation of Gi-coupled GPCRs causes an inhibition of the AC via G α_i and a decrease in the intracellular cAMP levels (Fig. 1A).^{1,2}

Since GPCRs comprise one of the largest gene families and ~26% of marketed drugs work by modulating GPCR

activation,^{3,4} quantification of intracellular cAMP levels remains an important methodology in molecular pharmacological studies of GPCRs. In recent years, biosensors that monitor intracellular cAMP levels have been developed, and the ongoing improvement of these assays provides several opportunities compared with previously employed assays,⁵ including the possibility to continuously measure the intracellular cAMP levels in real time. One such group of cAMP biosensors includes cAMP binding proteins fused to bioluminescent and/or fluorescent proteins for, respectively, Förster resonance

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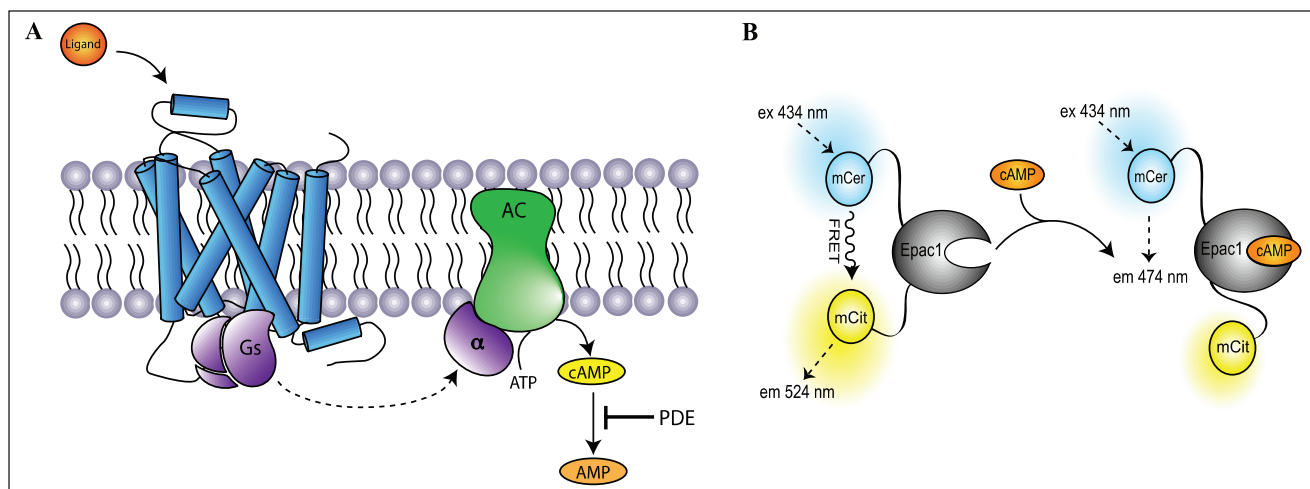


Figure 1. (A) Cartoon of the G protein–coupled receptor (GPCR) activation principle. Activation of a Gs-coupled GPCR results in dissociation of guanosine-5'-triphosphate (GTP)–bound G α s from the receptor, which stimulates the adenylate cyclase (AC) to form cyclic adenosine monophosphate (cAMP). Once formed, cAMP is quickly degraded to adenosine monophosphate (AMP) by specific phosphodiesterases (PDEs). PDE inhibitors prevent the degradation of cAMP, resulting in accumulation of cAMP. (B) The Epac149 biosensor is flanked by mCerulean (mCer) and mCitrine (mCit) on either side. Binding of cAMP results in a conformational change within the cAMP binding protein, “exchange protein directly activated by cAMP” (Epac). As a result, the distance between the two fluorescent proteins increases, which leads to a decrease in Förster resonance energy transfer (FRET) between mCer and mCit. A real readout signal is presented in our previous work.⁹

energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) measurement.^{6,7}

High-throughput screening (HTS) enables testing of large libraries of compounds for activity at a given target. Besides a relevant assay readout, the assays for HTS have additional requirements. To be compatible with automation, miniaturization, and a suitable plate reader, an HTS assay should be amenable for 96, 384, or 1536 microplate formats and have a good signal-to-noise ratio, which is stable over the time it takes to quantify the readout with a suitable plate reader. An inexpensive and preferably homogenous assay format with minimal handling further adds to the HTS assay usability.⁸

In previous work, we optimized a cAMP biosensor consisting of the Epac1 (exchange protein directly activated by cAMP) cAMP binding protein, flanked by yellow and cyan fluorescent proteins on either side with a more efficient FRET pair of fluorescent proteins.⁹ During the characterization of the cAMP biosensor, we observed that the readout-obtained format was surprisingly stable and reproducible and thus possibly amendable for HTS. Here we describe the development of a FRET-based HTS assay for identification and characterization of Gs-coupled GPCR ligands and PDE inhibitors in live cells.

The β_2 -adrenergic receptor (β_2 AR) was used as a representative of a Gs-coupled GPCR receptor. Overexpression of GPCRs results in saturation of the cellular desensitization capacity and thus continuous G protein coupling, reflected by prolonged downstream signaling.^{10–13} Prolonged signaling

may be preferable in HTS applications, and we therefore characterized two cell lines with different expression levels of the β_2 AR for comparison.

The β_2 AR can form an active-state complex without an agonist, a phenomenon also known as constitutive or basal activity. This agonist-independent activity can, however, be inhibited by inverse agonists. Drugs that inhibit the constitutive activity might be more effective therapeutics than neutral antagonists, and the interest for these types of compounds have been growing.¹⁴ In the cell line overexpressing the β_2 AR, accumulation of cAMP due to constitutive activity was evident when the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added, which we exploited to develop assay conditions compatible with identification of β_2 AR inverse agonists and PDE inhibitors. As described above, specific PDE inhibitors prevent the natural degradation of cAMP into AMP, resulting in increased cAMP concentrations. The various PDE isoforms are activated by a number of different mechanisms and are involved in numerous cellular processes due to their ability to hydrolyze and thus control the breakdown of the second-messenger signaling molecules cAMP and cyclic guanosine monophosphate.¹⁵ The cAMP PDE enzymes play a crucial role in regulating temporal and spatial changes in intracellular cAMP concentrations and have been used as important therapeutics for decades.^{5,16}

Although FRET-based cAMP biosensors were developed a decade ago,^{17–19} they have not previously been applied in an HTS setting. As described above, we recently

developed an optimized cAMP biosensor,⁹ and here we demonstrate that this biosensor shows great reproducibility and signal stability when used with overexpressed G_s-coupled GPCRs, enabling cAMP biosensor-based HTS.

Materials and Methods

Materials

Isoproterenol, forskolin, ICI 118-551 (ICI), epinephrine, nor-epinephrine, salbutamol, salmeterol, ritodrine, fenoterol, alprenolol, glucagon, oxyntomodulin, GLP-1-(7-36), rolipram, zaprinast, caffeine, HEPES, IBMX, magnesium and calcium chloride, bovine serum albumin (BSA), and Whatman glass microfiber filters (grade GF/B) were purchased from Sigma-Aldrich (Broendby, Denmark). Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), zeocin, geneticin G418, and penicillin/streptomycin were purchased from Invitrogen (Naerum, Denmark). Black 384-well plates were purchased from Greiner Bio-One (Kremsmünster, Austria). [³H] dihydroalprenolol and EnVision Xcite multilabel plate reader were from PerkinElmer (Waltham, MA).

Methods

cAMP FRET biosensor construct. The cAMP FRET biosensor consists of Epac1 fused with mCerulean (mCer) in the amino terminal and mCitrine (mCit) in the carboxy terminal. Binding of cAMP to Epac1 results in a conformational change within Epac1 by which the distance between the two fluorescent proteins increases and leads to a decrease in FRET. Generation of the construct has previously been described in Mathiesen et al.⁹ In short, the engineered expression vector for FRET biosensors (pcDNA3.1(+)-zeo-mCer-MCS-mCit) was generated by sequential subcloning of PCR-amplified DNA encoding mCerulean and mCitrine into a small multiple cloning sequence (MCS) of pcDNA3.1(+)-zeo. Complementary DNA (cDNA) encoding Epac1 amino acids 149 to 881 was then amplified and subcloned in between the two fluorescent proteins using the MCS.

Cell culture and expression of the cAMP biosensor in HEK293 cells. The β_2 AR used as a representative G_s-coupled GPCR is endogenously expressed in native HEK293 cells. The cAMP FRET biosensor construct was therefore stably expressed in HEK293 cells using the calcium phosphate transfection method as described in Mathiesen et al.⁹ The cAMP FRET biosensor cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C with 5% CO₂. Zeocin at 60 μ g/mL was used for selection and maintenance of the stably transfected cells.

Overexpression of the β_2 adrenergic receptor. To enhance the β_2 AR signaling, the β_2 AR was recombinantly overexpressed in

the parent Epac149 biosensor cell line by transfecting cells with a plasmid encoding the human β_2 AR in pcDNA3.1(+)-neo by the calcium phosphate transfection method. To obtain stably transfected cells, G418 at 500 μ g/mL was used for selection, while zeocin at 60 μ g/mL and G418 at 500 μ g/mL were used to maintain the cells. The β_2 AR-overexpressing Epac149 biosensor cell line will be referred to as the β_2 AR Epac149 biosensor cell line or the overexpressing cell line.

Determination of β_2 AR expression levels by saturation binding. Expression levels of β_2 AR in the native and overexpressing cell lines were determined by whole-cell [³H] dihydroalprenolol ([³H]DHA) saturation binding. Adherent cells grown in T175 flasks or 150-mm dishes to approximately 80% to 90% of confluence were detached by removing growth medium, and rinsing with DPBS once (no Ca²⁺ and no Mg²⁺), followed by incubation with DPBS supplemented with 2 mM EDTA for approximately 5 min or until detachment of cells. Cells were spun down at 1000 rpm for 5 min in a swinging bucket centrifuge; meanwhile, 10- μ L single-cell suspension cells were counted using a hemocytometer. The supernatant was carefully removed and the cells resuspended to 1.0×10^6 cells/mL in assay buffer consisting of HBSS (without Mg²⁺ and Ca²⁺) supplemented with 20 mM HEPES, 1 mM MgCl₂, 1 mM CaCl₂, and 0.2% BSA.

Saturation binding was carried out in a total volume of 500 μ L with 500,000 cells/tube for native expressing cells and 30,000 cells/tube for β_2 AR-overexpressing cells in assay buffer to minimize the potential ligand depletion. Cells, native or β_2 AR-overexpressing, were incubated with 0.12 to 7.5 nM [³H]DHA for 3 h on ice and terminated by three washes of 2 mL of the above HBSS buffer. Nonspecific binding was determined in the presence of 10 μ M cold alprenolol. Total and nonspecific binding as well as the exact concentrations of [³H]DHA used were quantified by liquid scintillation counting for total and nonspecific binding after overnight incubation of filters in UltraGold (Perkin Elmer, Denmark) scintillation fluid. Determinations were made in duplicates and counts recalculated to the number of receptor binding sites.

Measurement of G_s-coupled GPCR activation using the cAMP biosensor. In vitro experiments were performed on HEK293 cells stably expressing the cAMP FRET biosensor and either endogenously or recombinantly overexpressed β_2 AR. Cells were prepared as described above and resuspended at 750,000 cells/mL in assay buffer. The cells were left in the dark while preparing ligand solutions. For agonist screening, ligand solutions were prepared in two times the final concentration in assay buffer, and 20 μ L ligand solution was added to a black 384-well plate. Then, 20 μ L cell suspension (corresponding to 15,000 cells/well) was added by hand or with the EnVision pump (PerkinElmer). The plate was read continuously immediately after adding the cells on

an EnVision plate reader, measuring the fluorescence of mCer (474 nm) and mCit (524 nm) upon mCer excitation (434 nm).

Detection of inverse agonists. To detect inverse agonism, the PDE inhibitor IBMX was added to the assay buffer to induce cAMP accumulation in the receptor-unstimulated condition. A cell suspension with 750,000 cells/mL of β_2 AR Epac149 biosensor cells was prepared as described previously. The inverse agonist ICI 118-551 (ICI) was prepared in 20 μ M (corresponding to two times the final concentration) in assay buffer with 100 μ M IBMX. Then 20- μ L ligand solution and 20- μ L cell suspension (corresponding to 15,000 cells/well) were added to a black 384-well plate, and the plate was read continuously on an EnVision plate reader as described above.

Detection of phosphodiesterase inhibitors using the cAMP biosensor. For PDE inhibitor characterization, IBMX, rolipram, caffeine, and zaprinast were prepared in serial dilutions (two times the final concentration) in assay buffer. A cell suspension with 750,000 cells/mL of β_2 AR Epac149 biosensor cells was prepared as described above. Then, 20 μ L PDE inhibitor and 20 μ L cell suspension were added to a black 384-well plate, and the plate was read on an EnVision plate reader immediately after adding the cells.

Data analysis. Data were analyzed and presented using GraphPad Prism (GraphPad Software, San Diego, CA). In principle, the FRET ratio decreases upon cAMP accumulation, but to obtain graphs that correlate positively with cAMP concentrations, the FRET ratios were calculated as the ratio between the fluorescence of mCerulean and mCitrine.

Z factors was calculated as $Z = 1 - [(3 \times \text{SD}_{\text{sample}} + 3 \times \text{SD}_{\text{control}}) / (\text{mean}_{\text{sample}} - \text{mean}_{\text{control}})]$ as described by Zhang et al.²⁰

Results and Discussion

Assay Characterization and Detection of Gs-Coupled GPCR Activation

The assay presented here allowed measurement of Gs-coupled receptor activation through quantification of the intracellular cAMP levels in live cells. Agonist activation of the β_2 AR results in Gs-mediated adenylate cyclase activation and increased intracellular cAMP levels (**Fig. 1A**). Binding of cAMP to the Epac1 part of the biosensor induces a conformational change within the Epac1 protein, leading to an increased distance between the fluorescent proteins of FRET pair (**Fig. 1B**) and a decreased FRET signal. Two Epac149 biosensor HEK293 cell lines with different expression levels of the β_2 AR were used in the initial studies to establish the effect of receptor overexpression on

biosensor signal magnitude and duration. The low-expressing β_2 AR cell line expressed the β_2 AR endogenously, whereas the high-expressing β_2 AR cell line was obtained by stable overexpression of β_2 AR. Expression levels of the two cell lines were determined by [³H]DHA saturation binding (**Fig. 2A**). The low-expressing cell line displayed a B_{max} corresponding to $19,800 \pm 4,500$ binding sites/cell, whereas the high-expressing cell line had a B_{max} of $900,000 \pm 30,000$ binding sites/cell. The K_d values of the low- and high-expressing cell lines were averaged to 1.02 ± 0.37 nM and 0.61 ± 0.20 nM (mean $\pm$ SEM), respectively (no significant difference, Student *t* test). Thus, there was a 45-fold difference in β_2 AR expression levels between the two cell lines.

To determine the effect of the receptor expression level on the intracellular cAMP kinetics on agonist activation, the low and high β_2 AR-expressing cell lines were stimulated with different reference agonists. Isoproterenol (ISO) at 10 μ M produced a substantial increase in the cAMP level in both cell lines over time (**Fig. 2B**) that decayed significantly within 120 min for the low-expressing cell line. Forskolin, a direct activator of AC, also produced a significant increase in intracellular cAMP. The maximal forskolin response was higher than that produced by ISO, suggesting that full β_2 AR activation did not reach cAMP levels that saturated the dynamic range of the cAMP biosensor (**Fig. 2B**).

Both cell lines were subsequently stimulated with the β_2 AR agonists ISO, salbutamol, salmeterol, fenoterol, ritaline, epinephrine (Epi), and norepinephrine (NE) to generate concentration-response curves (**Fig. 2C,D**). The time point at which maximal cAMP levels were reached (12.5 min) was used for concentration-response curve generation. In the low-expressing cell line, ISO, Epi, NE, and fenoterol produced similar maximal responses, whereas salbutamol and salmeterol only activated the receptor partially (**Fig. 2C**), in agreement with previously published data.^{21,22} In contrast, all agonists produced similar maximal responses (**Fig. 2D**) in the high-expressing cell line due to overexpression of the β_2 AR. Potencies were determined in both cell lines and were found to be approximately 100-fold more potent in the high-expressing cell line. The rank order of agonist potencies in the cAMP assays of both the low- and high-expressing cell lines was found to correlate with each other and with the profile of β_2 AR agonists in the literature.^{21,23}

To examine a potential difference in kinetics of ligands and between cell lines, the cAMP response kinetics were plotted at the concentration at which signal saturation was reached (**Fig. 2E, F**). In the parent Epac biosensor cell line with low native β_2 AR expression, a fast and transient cAMP response was observed upon agonist-mediated receptor activation, and there were no significant differences in the cAMP response kinetics between the different β_2 AR agonists (**Fig. 2E**). In the β_2 AR Epac biosensor cell line, all agonists induced an immediate and prolonged cAMP signal (**Fig. 2F**) that did not return to the basal level as seen for the

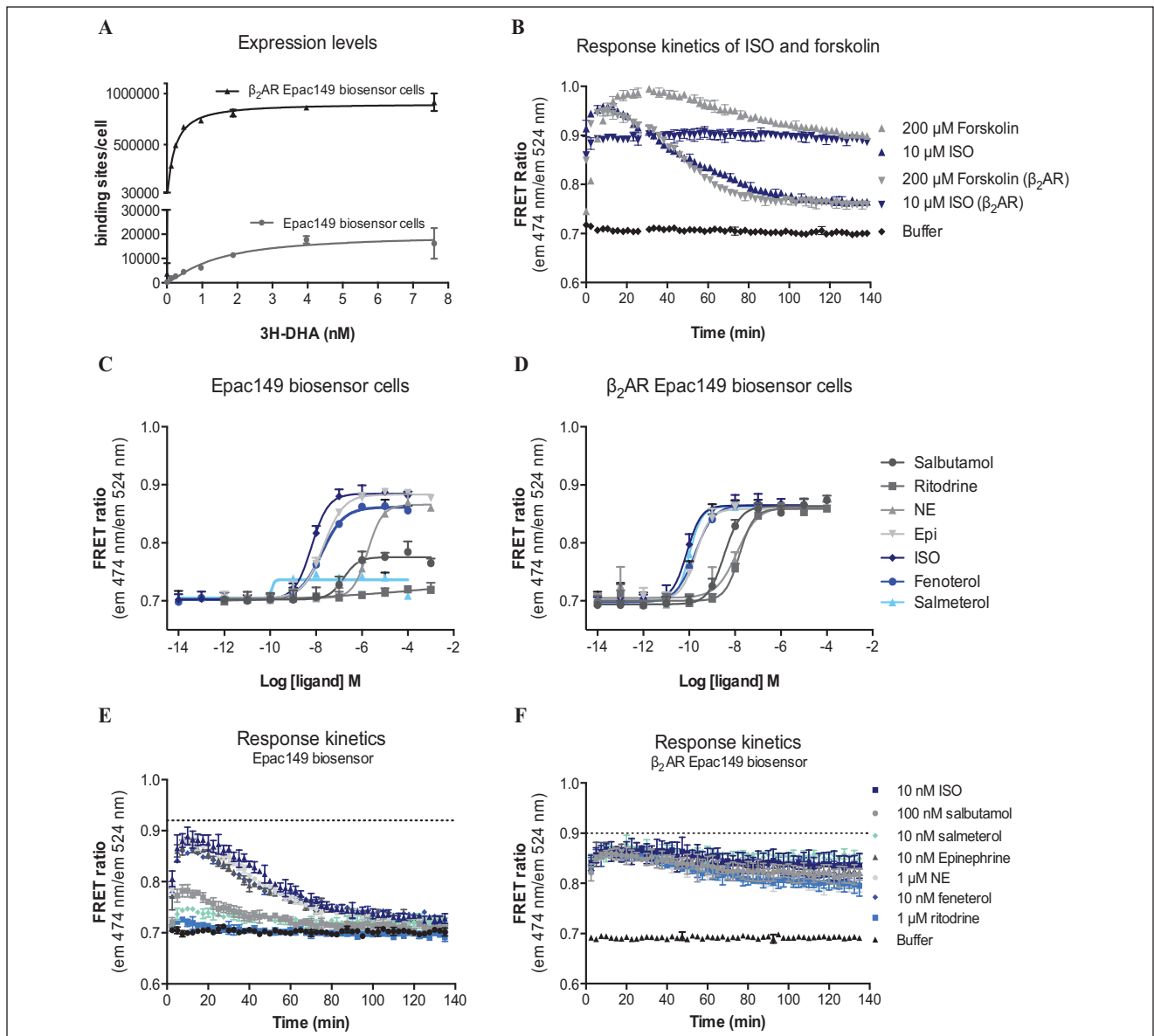


Figure 2. (A) Expression levels were determined by [³H]DHA binding. The β₂-adrenergic receptor (β₂AR) Epac biosensor cell line displays a significantly higher level of binding sites compared with the parent Epac biosensor cell line with endogenous β₂AR expression levels. Whereas the K_d values of the parent and β₂AR-overexpressing cell lines from multiple experiments averaged to 1.02 ± 0.37 nM and 0.61 ± 0.20 nM (mean ± SEM), respectively, they are 1.40 ± 0.75 nM and 0.22 ± 0.02 nM, respectively, in the above depicted experiment. (B) Response kinetics of isoproterenol (ISO) and forskolin in the parent and the β₂AR-overexpressing Epac biosensor cell lines. Forskolin produces a higher level of cyclic adenosine monophosphate (cAMP) than that by ISO in both cell lines. (C, D) Concentration-response relationships of ISO, fenoterol, epinephrine (Epi), norepinephrine (NE), ritodrine, salmeterol, and salbutamol in both cell lines. Native low β₂AR expression (Epac biosensor cells) enabled detection of partial agonism in contrast to the high-expressing cell line (β₂AR Epac149 biosensor cells) where all agonists produced a full maximum response. In the low-expression cell line, the pEC₅₀ values were as follows: ISO 8.2 ± 0.05, salmeterol n/a, fenoterol 7.8 ± 0.05, epinephrine (Epi) 7.7 ± 0.06, salbutamol 6.8 ± 0.20, norepinephrine (NE) 5.8 ± 0.06, and ritodrine n/a. In the high-expression cell line, the pEC₅₀ values were as follows: ISO 10 ± 0.07, salmeterol 10 ± 0.05, fenoterol 9.8 ± 0.06, Epi 9.7 ± 0.07, salbutamol 8.5 ± 0.07, NE 7.9 ± 0.14, and ritodrine 7.8 ± 0.07. (E, F) cAMP response kinetics in the Epac149 biosensor cell line and the β₂AR Epac149 biosensor cell line. In the native low-expression cell line, the agonist-induced response was fast and transient. ISO, Epi, NE, and fenoterol almost increased the cAMP level to the same level as forskolin (indicated by a dashed line), whereas salbutamol, salmeterol, and ritodrine caused only a slightly increase in the cAMP level, indicating partial agonism of these ligands. In the β₂AR-overexpressing cell line, all agonists produced a fast and prolonged cAMP level. FRET, Förster resonance energy transfer.

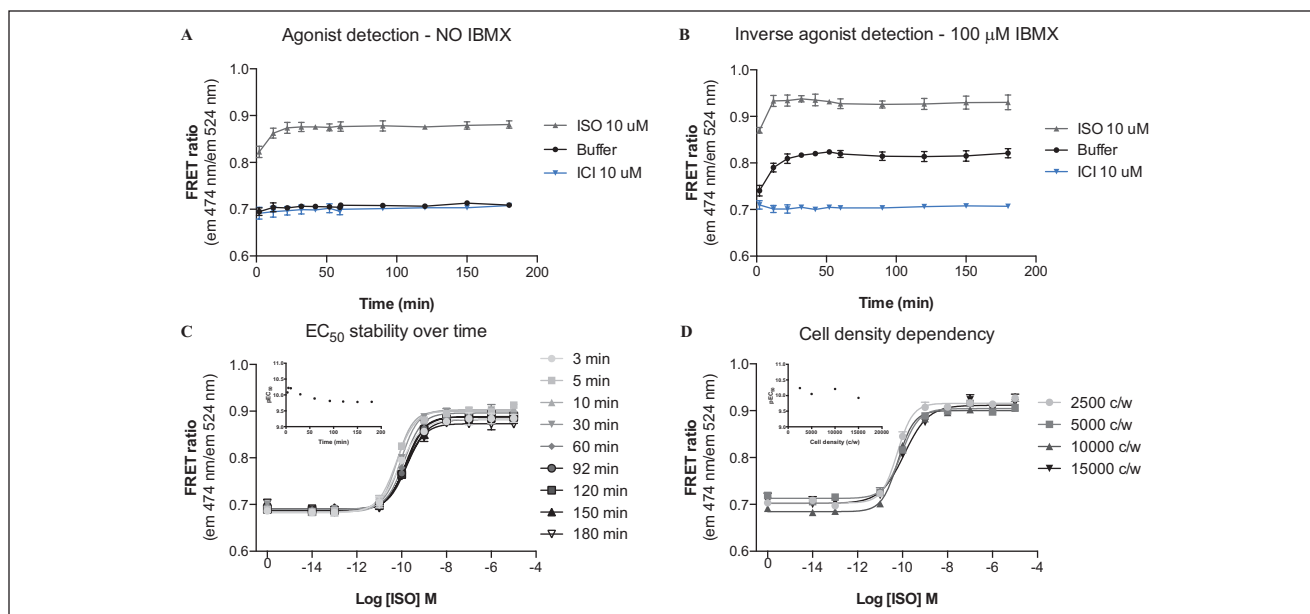


Figure 3. (A) Stimulation of the β_2 AR Epac biosensor cell line with isoproterenol (ISO), ICI 118-551 (ICI), and buffer revealed an applicable assay window for agonist detection. The window was stable over time. (B) Addition of 100 μ M 3-isobutyl-1-methylxanthine (IBMX) increased the basal cAMP levels, allowing inverse agonist detection. ICI efficiently decreased the cAMP levels. (C) To assess assay stability over time, the EC_{50} of ISO was determined at different time points after assay initiation using 10,000 cells/well. ISO dose dependently increased the Förster resonance energy transfer (FRET) ratio with an average EC_{50} of 0.12 nM (pEC_{50} of 9.9 ± 0.04). The pEC_{50} values were as follows: 3 min, 10 ± 0.05 ; 5 min, 10 ± 0.05 ; 10 min, 10 ± 0.04 ; 30 min, 10 ± 0.03 ; 60 min, 9.9 ± 0.04 ; 92 min, 9.8 ± 0.03 ; 120 min, 9.8 ± 0.04 ; 150 min, 9.8 ± 0.04 ; and 180 min, 9.8 ± 0.05 . The inset shows the variation in pEC_{50} values over time. (D) To examine the influence of cell density, the EC_{50} of ISO was determined after 10 min using different cell densities (average EC_{50} of 0.08 nM with a pEC_{50} of 10 ± 0.05). The pEC_{50} values were as follows: 2500 c/w, 10 ± 0.04 ; 5000 c/w, 10 ± 0.05 ; 10,000 c/w, 10 ± 0.04 ; and 15,000 c/w, 9.9 ± 0.08 . The inset shows the variation in pEC_{50} values as a function of the cell density.

parent Epac149 biosensor cell line in the measured time frame of 140 min. This phenomenon is most likely due to the presence of spare receptors in the high-expressing cell line. These results are consistent with previous studies, enlightening that receptor overexpression may cause a saturation of the desensitization capacity of the cell, resulting in prolonged downstream signaling.¹³ Another consequence of receptor overexpression was that partial agonism was masked. Salbutamol and even ritodrine, which produced only a negligible cAMP increase in the parent Epac149 biosensor cell line, appeared as full agonists in the overexpressing cell line (Fig. 2D). The desensitization capacity of the cell, on the other hand, was not saturated in the parent Epac149 biosensor cell line with low native β_2 AR expression, consistent with the transient cAMP production.

Based on the above comparison of the low- and high-expressing cell lines, it is clear that overexpression of the β_2 AR (1) enables detection of weak and/or partial agonists and (2) results in a sustained signal over time, which is highly advantageous in large HTS screens aimed at hit identification. On the other hand, endogenous expression levels allow for examination of desensitization kinetics and differentiation of full from partial agonists, which may be a problem in

general cAMP accumulation assays.⁵ The initial cAMP kinetics of agonist stimulation can, however, not be reliably quantified due to the fast onset of cAMP formation.

HTS Applicability

Overexpression of the β_2 AR resulted in a sustained signal over time, and even though the cAMP kinetics are far from the physiological response, these conditions are likely preferable in ligand screening setting. Consequently, only the high-expressing β_2 AR Epac biosensor cell line was further studied for its HTS applicability.

Detection of agonists and inverse agonists. To further explore conditions optimal for a stable and a sustained signal, the effect of IBMX, a known PDE inhibitor used for accumulating cAMP in relation to other cAMP detection principles, was tested. The effects of buffer, ISO, and the inverse agonist ICI on cAMP levels over 180 min were examined in the absence (Fig. 3A) and presence (Fig. 3B) of IBMX. IBMX at 100 μ M perturbed the cAMP turnover and increased unstimulated cAMP levels toward the stimulated ISO state, reflecting the constitutive activity of the β_2 AR.

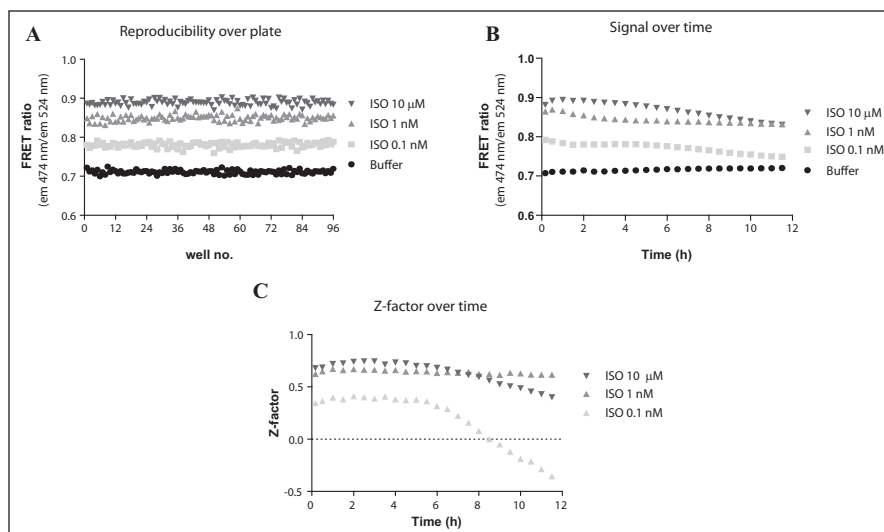


Figure 4. (A) Assay reproducibility over an assay plate (measured after 180 min). Buffer and isoproterenol (ISO; 0.1 nM, 1 nM, and 10 μ M) were added to each of 96 wells in a 384-well plate. (B) The signal over time and (C) Z factors were determined for up to 12 h. Z factors were calculated by $Z = 1 - [(3 \times SD_{\text{sample}} + 3 \times SD_{\text{control}}) / (\text{mean}_{\text{sample}} - \text{mean}_{\text{control}})]$. The two highest concentrations of ISO (10 μ M and 1 nM) had good Z factors above 0.5 for at least 9 h, whereas the lowest ISO concentration (0.1 nM) resulted in Z factor values around 0.4 for up to 5 h. FRET, Förster resonance energy transfer.

Without addition of IBMX, a large assay window that allowed agonist detection was obtained, and ISO produced a prominent increase in cAMP and thus a large change in the FRET ratio (Fig. 3A). Addition of the PDE inhibitor IBMX allowed the cAMP formed, due to constitutive activity, to accumulate and thereby enabled inverse agonist screening. Under these conditions, the inverse agonist ICI efficiently inhibited the cAMP levels below the basal level (Fig. 3B), confirming that the assay can also be applied for screening for inverse agonists.

Reproducibility of assay parameters over time. To generate reproducible data, functional assays often have limitations to assay parameters such as the time point at which the assay response is quantified and the amount of cells used. To test the assay stability over time, ISO concentration-response curves and EC_{50} values were examined at different time points and at different cell densities.

The EC_{50} of ISO was determined at nine different time points ranging from 3 to 180 min to test the assay stability over time (Fig. 3C). Only small variations were observed, and the average EC_{50} value was determined to 0.12 nM with a pEC_{50} value of 9.9 ± 0.04 . Over time, there was a tendency toward a minor right shift of the ISO concentration-response curves with the EC_{50} increasing from 0.08 nM to 0.17 nM (twofold) over 3 h.

The EC_{50} of ISO was also determined at four different cell densities covering a sixfold difference in cell densities ranging from 2500 cells/well to 15,000 cells/well (Fig. 3D). Only minor variations in the concentration-response curves were observed, with an average EC_{50} of 0.08 nM ranging from 0.06 to 0.12 nM with a pEC_{50} of 10 ± 0.05 , also corresponding to a twofold change. Unlike most other functional assays, the cAMP biosensor FRET assay is less dependent on the cell density between assays. Many functional assays rely on

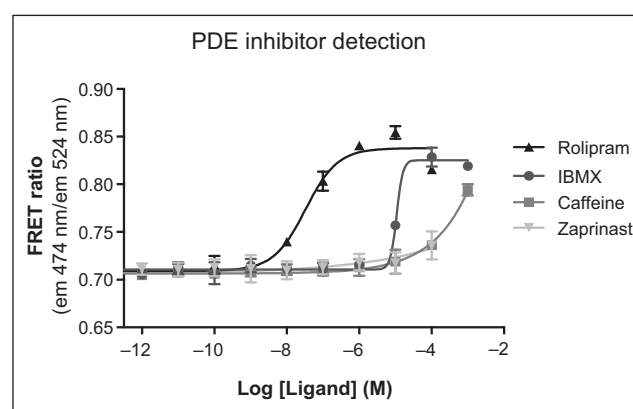


Figure 5. Concentration-response curves of four phosphodiesterase (PDE) inhibitors (3-isobutyl-1-methylxanthine [IBMX], rolipram, caffeine, and zaprinast) in β_2 AR Epac149 biosensor cells. Full curves were obtained with rolipram and IBMX, whereas only the highest concentrations of caffeine and zaprinast were able to induce changes in Förster resonance energy transfer (FRET).

quantification of the absolute levels of the second messenger formed by a specific number of cells. Because the cAMP biosensor FRET readout is quantified by FRET in each single cell by the unimolecular cAMP biosensor, the assay is less dependent on the cell density. For the FRET cAMP biosensor, the limitation on cell density is rather due to the plate reader sensitivity range (too high or too low signal) for the fluorescence protein intensity determination used to calculate the FRET ratio.

The assay is thus stable over a range of time points and cell densities, which is an advantage when using the assay in an HTS campaign. Although we here described how to use cells in suspension, similar results are also observed for adherent cells (data not shown). If necessary, the cAMP

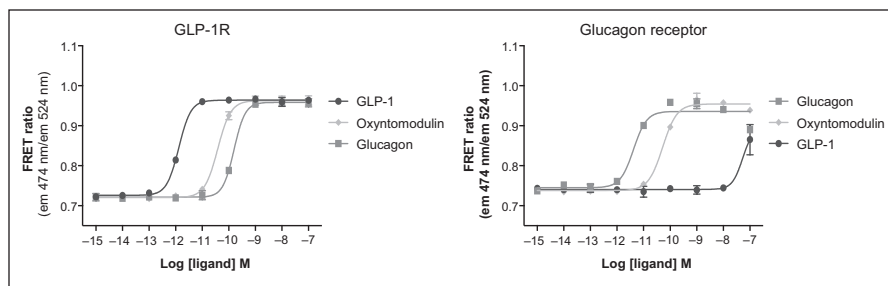


Figure 6. Concentration-response curves of glucagon-like peptide-1 (GLP-1), oxyntomodulin, and glucagon in cells expressing the Epac149 biosensor and either the glucagon-like peptide 1 receptor (GLP-1R) or the glucagon receptor. FRET, Förster resonance energy transfer.

concentrations can be calculated via a standard curve as described in Mathiesen et al.⁹

Assay reproducibility and Z factors. The assay reproducibility was assessed by determination of the Z factor at optimal and more challenged conditions since the signal-to-noise ratio appeared to be stable for over 3 h when stimulated with a full agonist at a 10 μ M saturable concentration (**Fig. 3A**). The Z factor determination was thus determined 1) continuously for 12 h after initiation and 2) at different ISO concentrations corresponding to EC_{100} , EC_{80} , and EC_{40} cAMP levels. The assay was reproducible (**Fig. 4A**) and the signals stable for at least 6 h (**Fig. 4B**). The calculated Z factors were correspondingly good and stable for at least 8 h (**Fig. 4C**). However, at the lower EC_{40} ISO concentration, the Z factor was ~ 0.4 for the first 6 h, after which it rapidly declined.

Phosphodiesterase Inhibitor Detection

Phosphodiesterase inhibitors have been used as important therapeutics as well as tool compounds for decades.¹⁶ As mentioned previously, PDEs degrade cAMP by catalyzing the hydrolysis of the 3'ester bond to form AMP. During assay optimization using IBMX to accumulate cAMP (**Fig. 3B**), we hypothesized that the Epac1 biosensor might also be used to identify and characterize PDE inhibitors in addition to Gs-coupled GPCR ligands.

To test this, four PDE inhibitors were tested in the β_2 AR Epac biosensor cell line. IBMX and rolipram both produced dose-dependent inhibition of the cAMP degradation and thus an increase in the cAMP levels (**Fig. 5**), whereas only the highest concentrations of caffeine and zaprinast led to increased cAMP levels. These differences are most likely due to the subtype selectivity of the PDE inhibitors in relation to the PDEs expressed in HEK293 cells. The results indicate that the β_2 AR Epac biosensor cell line can also be used for detection of PDE inhibitors. Likely, in the Epac biosensor cell line, the overexpressed constitutive active β_2 AR continuously generates cAMP, which is continuously degraded by PDEs. Therefore, perturbation of the constant steady-state cAMP level (**Fig. 3A**) by a PDE inhibitor results in a new increased cAMP steady-state level (**Fig. 3B**). This difference

in cAMP levels by PDE inhibitors may thus be used for identification and characterization of PDE inhibitors.

cAMP Epac Biosensor and Other GPCRs

In this study, the β_2 AR was used as a representative Gs-coupled receptor, although the Epac biosensor may also be expressed with other GPCRs to detect Gs signaling. The glucagon-like peptide 1 receptor (GLP-1R) and glucagon receptor, which both increase cAMP levels in HEK293 cells when activated, were stably expressed in the native Epac biosensor cell line as described for the β_2 AR. The agonists GLP-1(7-36), oxyntomodulin, and glucagon all produced dose-dependent increases in the FRET ratio according to their known pharmacology in the respective cell lines (**Fig. 6**), verifying that the biosensor may be used with other Gs-coupled GPCRs. A similar increase in cAMP response upon agonist stimulation has previously been observed in HEK293 cells expressing the Epac biosensor and the GLP-1R.²⁴

In conclusion, we here show that the FRET-based cAMP biosensor cell line overexpressing a Gs-coupled receptor can be used for HTS. The assay is amenable for the 384-well plate format and does not require any costly reagents to quantify the intracellular cAMP levels. The readout is robust, largely independent of cell density, reproducible with good Z factors, and stable for at least 8 h. Overall, the presented assay is highly amenable for HTS applications and hit confirmation of Gs-coupled GPCR agonists, PDE inhibitors, and other GPCR ligand classes such as inverse agonists.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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