CHAPTER THREE

Real-Time Measurement of Cannabinoid Receptor-Mediated cAMP Signaling

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Contents

1. Introduction 44
   1.1 Canonical Cannabinoid Receptors Are GPCRs 44
   1.2 Importance of cAMP Signaling 45
   1.3 cAMP Signaling of Cannabinoid Receptors 45
   1.4 Temporal Control of GPCR Signaling 46
   1.5 Types of cAMP Biosensor 47
2. Detecting Cytoplasmic cAMP Using CAMYEL 48
   2.1 Protocol 48
   2.2 Data Analysis 51
3. Design and Validation of V8-CAMYEL 52
   3.1 Validation of V8-CAMYEL 54
4. Summary 56
References 56

Abstract

Cannabinoid receptors, like other GPCRs, signal via a spectrum of related signaling pathways. Recently, monitoring GPCR-mediated cAMP signaling has become significantly easier with the development of genetically encoded, transfectable cAMP biosensors. Cell lines transfected with these biosensors can be monitored continuously, allowing the analysis of receptor-mediated signaling in unprecedented detail. Here, we describe a protocol for transfectable biosensors which report cellular cAMP concentrations by bioluminescence resonance energy transfer (BRET). This assay system has been utilized to elucidate the temporal nature of agonists and allosteric modulators of the cannabinoid receptor CB1. In particular, the CB1 allosteric modulator ORG27569 has been shown to modify receptor agonism in a time-dependent fashion; a characteristic which would not have been observed via traditional endpoint methods of detecting cAMP

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signaling. BRET cAMP biosensors are suitable for miniaturization and automation, and as such are valuable and cost-effective tools for moderate- to high-throughput experimental protocols.

ABBREVIATIONS

AUC area-under-the-curve
BRET bioluminescence resonance energy transfer
CAMYEL cAMP sensor using YFP-Epac-Rluc
cAMP cyclic adenosine monophosphate
CB1 cannabinoid receptor 1
CB2 cannabinoid receptor 2
EPAC exchange proteins activated by cAMP
FRET Förster resonance energy transfer
GPCR G protein-coupled receptor
HBSS Hank's-buffered saline solution
PBS phosphate-buffered saline
PDE phosphodiesterase
PEI polyethyleneimine
PKA protein kinase A
V8-CAMYEL Venus-Rluc8 cAMP sensor using YFP-Epac-Rluc

1. INTRODUCTION

1.1 Canonical Cannabinoid Receptors Are GPCRs

The effects of cannabinoid ligands are mediated through at least two G protein-coupled receptors (GPCRs). The canonical cannabinoid receptors are cannabinoid receptor 1 (CB1) and 2 (CB2), which are most abundant in the central nervous system (Howlett et al., 2004) and in cells of the immune system (lymphocytes, monocytes, and microglia; Howlett et al., 2004; Klein et al., 2003), respectively. Furthermore, there is evidence for at least one more cannabinoid receptor; current candidate receptors for "CB3" include the GPCRs GPR18 and GPR55 (Pertwee et al., 2010).

GPCRs are a large family of transmembrane proteins. As their name suggests, GPCRs activate G proteins, which are heterotrimeric structures containing α, β, and γ subunits (Dupré, Robitaille, Rebois, & Hébert, 2009). Once activated, the cellular effects of G proteins depend on the particular G protein subtype and cellular environment of the receptor/G protein system. There are four groups of G proteins, traditionally defined by their Gα subunit. The signaling profiles of the various Gβγ subunits are less well characterized, but these also affect a wide range of effectors (Altier, 2012;

Currie, 2010; Doupnik, 2008; Dupré et al., 2009; Huang, Slesinger, Casey, Jan, & Jan, 1995).

Activated GPCRs can also recruit arrestins, a step which has previously been considered as a stage in the desensitization sequence (Magalhaes, Dunn, & Ferguson, 2012). However, arrestins have been demonstrated to initiate some signaling, including activation of multiple MAPK pathways (Luttrell & Lefkowitz, 2002; Zhan, Kook, Gurevich, & Gurevich, 2014). This provides a second complement of GPCR signaling cascades beyond canonical G protein effects.

1.2 Importance of cAMP Signaling

Adenylate cyclases catalyze the formation of cyclic adenosine monophosphate (cAMP), and are stimulated by Gαs and inhibited by Gαi G protein subunits. In addition, different adenylate cyclase subtypes are regulated by Gβγ and Ca^{2+} (Hanoune & Defer, 2001; Kamenetsky et al., 2006). cAMP is a key regulator of cellular metabolism and modulates several cellular processes differentially depending on cAMP concentration. Protein kinase A (PKA) activity is saturated by low cAMP concentrations (1 μM) (Antoni, 2012); activation of PKA leads to the phosphorylation of a wide variety of protein targets (Logue & Scott, 2010; Taylor et al., 2004). Two types of ion channel are activated by cAMP: hyperpolarization-activated and cyclic nucleotide-modulated channels, and channels directly modulated by cyclic nucleotides. These channels are maximally responsive to 10 μM cAMP, and when activated they increase the electrical activity of the cell (Antoni, 2012). Exchange proteins activated by cAMP (Epac) require high levels of cAMP (40 μM), and in turn activate Rap pathways, which can affect processes including phospholipase activity and cytoskeletal changes (Gloerich & Bos, 2010; Raaijmakers & Bos, 2009).

Phosphodiesterases (PDEs) reduce cellular cAMP concentrations by hydrolyzing it into 5′AMP. Some PDEs are also regulated by cAMP, with the cAMP-binding sensitivity varying significantly between PDE subtypes (Keravis & Lugnier, 2010).

1.3 cAMP Signaling of Cannabinoid Receptors

Both CB1 and CB2 are primarily classified as Gαi-coupled receptors (Felder et al., 1995; Howlett, Qualy, & Khachatrian, 1986; Matsuda, Lolait, Brownstein, Young, & Bonner, 1990; Munro, Thomas, & Abu-Shaar, 1993), with a signaling profile which, on the whole, reflects the canonical

Gαi pathways. The traditional test for Gαi dependence in a cell signaling assay is to pretreat cells with pertussis toxin. This ADP-ribosylates the Gαi subunit, preventing Gαi turnover (Milligan, 1988). Using this approach, both CB1 and CB2 have been shown to inhibit the activity of adenylate cyclases, thus reducing cAMP production, via activation of Gαi proteins (Bouaboula et al., 1996; Matsuda et al., 1990).

Although CB1 is typically described as a Gαi-linked GPCR, there is robust evidence that CB1 is capable of activating non-Gαi-type signaling pathways. The most frequently reported "secondary" signaling pathway of CB1 is through activation of cAMP, likely through Gαs, first reported in medium spiny neurons (Glass & Felder, 1997). This signaling pathway can be unmasked by application of pertussis toxin, which prevents Gαi signaling (Bonhaus, Chang, Kwan, & Martin, 1998; Felder et al., 1998; Glass & Felder, 1997; Kearn, Blake-Palmer, Daniel, Mackie, & Glass, 2005; Scotter, Goodfellow, Graham, Dragunow, & Glass, 2010). To date, few other experimental designs have demonstrated CB1-mediated cAMP accumulation in untreated cells. For example, WIN55,212-2 gave a Gαs-like response when applied to rat globus pallidus cells (Maneuf & Brotchie, 1997), as did desacetyllevonantradol when applied to a neuroblastoma cell line (Bash, Rubovitch, Gafni, & Sarne, 2003), and CP55,940 applied to human trabecular mesh cells (Stamer et al., 2001). Additionally, Gαq (Lauckner, Hille, & Mackie, 2005; McIntosh, Hudson, Yegorova, Jollimore, & Kelly, 2007), Gα12/13 (Ishii & Chun, 2002), and Gαz (Garzon, de la Torre-Madrid, Rodriguez-Munoz, Vicente-Sanchez, & Sanchez-Blazquez, 2009) coupling by the CB1 receptor have also been reported.

1.4 Temporal Control of GPCR Signaling

Several cellular signaling pathways exercise control over the spatial and temporal profile of GPCR-mediated cAMP signaling (reviewed extensively, e.g., by Caldieri & Sigismund, 2016; Ellisdon & Halls, 2016). These mechanisms can be investigated by using real-time analysis of signaling pathways. The use of such methods are also being driven by an increasing interest in characterizing functional selectivity—the ability of GPCR ligands to stabilize different receptor conformations, and thus elicit diverse balances of the various signaling pathways (Mallipeddi, Janero, Zvonok, & Makriyannis, 2016). Specifically, real-time analyses of cAMP signaling can and have been used for characterizing biased agonists (Redmond et al., 2016) and allosteric

modulators (Cawston, Connor, Di Marzo, Silvestri, & Glass, 2015; Cawston et al., 2013) of CB1.

1.5 Types of cAMP Biosensor

There are many options for measuring cAMP in biological samples. Biochemical detection methods, including radioimmunoassays and kit-based immunodetection assays (e.g., Hunter & Glass, 2015), lyse cells at an end point and analyze the final concentration of cAMP present in the lysate. Stimulating the cells in the presence of isobutylmethylxanthine (IBMX) prevents the degradation of cAMP, accumulating total cAMP during the assay incubation time. However, an inherent limitation of these end-point assays is the inability to see fluctuations in signaling profiles which are ultimately hidden in the final cAMP reading. More sophisticated cAMP assays are required if we wish to observe the complexity of these cAMP temporal signaling profiles.

The earliest iterations of cAMP sensors capable of real-time reporting were injectable, or multisubunit transfectable systems (reviewed in Sprenger & Nikolaev, 2013). These have now been surpassed by transfectable single-chain reporters, utilizing Förster resonance energy transfer (FRET, fluorescence-based) or bioluminescent resonance energy transfer (BRET). Both FRET and BRET are based on the transfer of energy from one fluorophore to another, based on physical proximity; binding of cAMP to these sensors moves the fluorophores, thus altering the efficiency of the energy transfer (Stryer, 1978; Wolber & Hudson, 1979; Wu & Brand, 1994).

Transfectable biosensors offer the opportunity to monitor cellular signaling profiles in real time. Many cAMP biosensors are available and are based on the cAMP-binding domains of PKA, cyclic nucleotide-gated channels, or Epac proteins (Nikolaev, Bünemann, Hein, Hannawacker, & Lohse, 2004; Norris et al., 2009; Ponsioen et al., 2004). There are a variety of FRET-based biosensors for cAMP (reviewed in Castro, Guiot, Polito, Paupardin-Tritsch, & Vincent, 2014), which are invaluable for microscopy-based assays. However, they are less useful for moderate- to high-throughput signaling assays, which require the analysis of dozens or hundreds of experimental conditions. Assays such as these greatly benefit from using BRET biosensors, which are unaffected by the complications of fluorophore bleaching and sample autofluorescence (Boute, Jockers, & Issad, 2002). Furthermore, BRET assays have proven easily amenable to miniaturization (i.e., scaling

to microtiter plates), and automation of both assay execution and analysis (Mo & Fu, 2016).

Here, we first describe the use of a cAMP sensor using YFP-Epac-Rluc (CAMYEL), which utilizes the Epac1 cAMP-binding domain to separate a BRET pair of wildtype Rluc and a Citrine YFP variant and has a moderate affinity for cAMP (K_d 8.8 μM) (Jiang et al., 2007). Later, we describe the design, validation, and use of a BRET biosensor with increased light output, in which Rluc is replaced with Rluc8, and the YFP variant with Venus (termed V8-CAMYEL).

2. DETECTING CYTOPLASMIC cAMP USING CAMYEL

2.1 Protocol

2.1.1 Equipment

Widefield microscope, preferably with fluorescence capabilities.

Plate reader capable of bioluminescence detection, e.g., VictorXLight (PerkinElmer), LumiStar (BMG LabTech). Optionally, this can include injector capabilities.

Single- and multichannel stepper pipettes.

2.1.2 Cell Line Considerations

Here, we describe the use of HEK293 cells (and lines derived from this parental line), transfected with plasmid DNA utilizing polyethyleneimine (PEI). HEK293 cells are commonly used in cell signaling assays and are amenable to transfection by a range of methods. PEI is a low-cost and reliable transfection reagent, making it ideal for frequent and large-scale experiments. However, any transfectable cell line can be substituted, grown in an appropriate media and transfected by any efficient method.

2.1.3 Reagents

HEK293 or an appropriate cell line (wildtype, or stably transfected with a receptor of interest).

Tissue culture reagents:

DMEM with 10% fetal bovine serum (before transfection, penicillin/streptomycin can be included in the growth medium; however, this should be removed before addition of the transfection reagents).

Trypsin–EDTA.

Phosphate-buffered saline (PBS).

150 mM NaCl.

Linear PEI, MW 25 kDa (Polysciences Inc., cat. 23,966, or equivalent)—dissolved to 1 mg/mL in 150 m*M* NaCl with gentle heating, stored in single-use aliquots and stored at −20°C (we do not find it necessary to filter sterilize these preparations).

pcDNA3L-His-CAMYEL (ATCC, cat. MBA-277), transfection-grade plasmid.

White 96-well plates, such as CulturPlate-96 (PerkinElmer, cat. 6,005,680).

Clear 96-well plates, suitable for microscopy.

Poly-L-lysine or poly-D-lysine.

Hank's balanced salt solution (HBSS).

Assay buffer: HBSS +1 mg/mL bovine serum albumin.

Coelenterazine h, 2400 μ*M* in ethanol (store at −20°C, protect from light) (Nanolight Technology, cat. 301, or equivalent).

Drugs:

Forskolin 10 m*M* in DMSO.

CP55,940 10 m*M* in ethanol, or other agonists.

Test compounds, such as allosteric modulators or antagonists.

2.1.4 Preparation of Cells—Seeding, Transfection, Reseeding of Adherent Cells

Day 1: Seed 5,000,000–10,000,000 HEK293 cells per 10 cm dish, in order to achieve 70% confluency the following day.

Day 2: Transfect cells

- Dilute 5 μg of CAMYEL plasmid in 250 μL of 150 m*M* sterile NaCl. In a second tube, dilute 30 μL of 1 mg/mL PEI stock in 220 μL of 150 m*M* NaCl (i.e., a 1:6 DNA:PEI ratio).
- Combine all the diluted DNA with all the diluted PEI, and immediately vortex very well for at least 3 s.
- Incubate the PEI/DNA mixture for 10 min.
- Just before the 10 min incubation is finished, replace media on cells with 8 mL fresh growth media.
- Add 500 μL PEI/DNA mixture per plate to media (gently, dropwise). Rock plates to mix and then return cells to the 37°C CO_2 incubator.

Day 3: Replate cells into microtiter plates.

- Treat white and clear 96-well plates with poly-L- or poly-D-lysine in order to facilitate cell adhesion. (Some cell lines will not require treated plasticware and will remain adherent throughout several wash steps.)

- Lift cells by trypsinization, resuspend in standard growth media, and seed into white 96-well plates (for BRET assay detection) and clear 96-well plates (for microscopy). Cells should be seeded at 25,000–60,000 cells/well, in order to achieve at least 70% confluency the following day.

2.1.5 Performing the Assay, Including Strategies for Seeing Gi vs Gs Signaling

- Inspect the cells in the clear 96-well plate, checking for cell health, confluency, and expression of CAMYEL (using the intrinsic fluorescence of the biosensor). Cells on this plate can be processed for immunocytochemistry (e.g., for receptor expression) if required.
- Prepare and assay 1–2 rows of wells at a time. Prolonged incubation times (>90 min) in assay media can alter cell signaling profiles, so it is advisable to prepare the rows in a staggered fashion. (See Note 1.)
- Using a vacuum aspirator, remove the growth media (containing phenol red), wash the cells once with 150 μL/well HBSS, then add 80 μL/well assay media. Equilibrate for 30 min, either in a 37°C CO_2 incubator or in the plate reader with temperature control (for HEK293 cells, either is acceptable).
- Prepare coelenterazine h at 10× concentration in assay buffer (i.e., 50 μ*M*, to be used at 5 μ*M*). Keep diluted coelenterazine h on ice, protected from light.
- Prepare drugs, including vehicle controls, at 10× concentration in assay buffer and store on ice. As CB1 and CB2 are Gαi-linked receptors, adenylate cyclase activity must first be raised in order to provide a window for observing inhibitory activity. Generally, this is done by coincubation with forskolin, which is an agonist of adenylate cyclase (Seamon, Padgett, & Daly, 1981). Cell lines vary in their sensitivity to forskolin and so a concentration–response curve should be generated to choose a suitable concentration; we find 5–10 μ*M* is appropriate for HEK293 cells.
- Incubate cells with coelenterazine h: Add 10 μL/well of 10× coelenterazine h into the assay buffer of every well, and agitate plate to mix (manually, or using a gentle shake function in the plate reader). Incubate for 5 min inside the plate reader (with temperature control held at 37°C). We recommend measuring the luminescence during this time, to monitor the diffusion of the coelenterazine h substrate into the cells.

- Add drugs to cells: Add 10 μL/well of 10 × drugs into the assay buffer (therefore, total volume per well is 100 μL), and agitate plate to mix, and start measuring the luminescence immediately. (See Note 2.)
- Luminescence measurements (0.25–1 s per well) should be made at 460 nm (Rluc) and 535 nm (YFP), using a suitable bandwidth (25 nm, for example). Using this protocol for CAMYEL cAMP detection, biosensor data can be gathered for at least 30 min before the signal-to-noise ratio becomes unfavorable (primarily due to consumption of the coelenterazine h). (See Note 3.)

Notes:

1. Up to a whole 96-well plate can be measured in a single assay run, provided that the plate reader settings allows either rapid, sequential detection of luminescence in each well, or simultaneous detection of both 460 and 535 nm wavelengths (possible in some luminometers, including the BMG LumiStar). The longer the time between reads of the same well, the less refined the time course data will be.
2. If the injector functions of the plate reader are used, it is important to ensure that the injector lines are made of a low-binding plastic suitable for cannabinoids.
3. If time courses beyond 30 min are required, additional coelenterazine h can be added to the samples during the assay.

2.1.6 Cell Suspension Assay Variation

Do not replate cells on day 3. On day 3 or 4, lift adherent cells by EDTA or scraping (not trypsin, which would cleave cell surface proteins). Remove the EDTA and growth media from the cells by centrifugation, and resuspend in assay buffer. After determining the cell concentration, distribute the cells into 96- or 384-well white plates and perform a BRET assay immediately.

2.2 Data Analysis

The most intuitive way to display the activity of the CAMYEL biosensor is by plotting the inverse BRET ratio as a function of time, as shown in Fig. 1. The inverse BRET ratio is calculated by dividing the donor signal (460 nm) by the acceptor signal (535 nm), such that higher BRET ratio reflects more cytoplasmic cAMP concentration. Normalization can be performed, for example by setting the "basal" inverse BRET ratio as 0% and forskolin to 100%, which can be particularly useful in comparing between experiments performed on different plate readers or cell lines, which can give different baseline readings. Graphing, curve fitting and most statistical analysis can

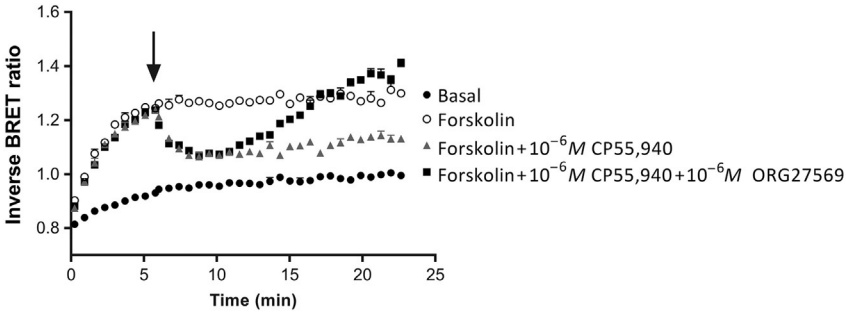

Fig. 1 HEK293 cells were treated with vehicle (for basal samples) or forskolin at 0 min, and CP55,940 ± ORG27569 was added to the appropriate samples at approximately 5 min (indicated by *arrow*). Biosensor activity is shown on the vertical axis, plotted as a nonnormalized inverse BRET assay.

be performed by a program such as Prism (GraphPad). Area-under-the-curve (AUC) analysis provides results most similar to a traditional cumulative cAMP assay (see Fig. 2), albeit with the obvious shortcoming of removing the time-course data. However, it is also useful to inspect the raw data (as graphed in Fig. 1) for deviations from single-phase signaling profiles.

As an example, Fig. 1 shows that the CB1 allosteric modulator (ORG27569), when applied with an agonist (CP55,940), mediates a two-phase cAMP response. In this assay design, forskolin was added first, and then at 5 min (at nearly the plateau of the forskolin response) the other ligands were added together to the appropriate wells. The results demonstrate a strikingly different time-response trace when compared to the one-phase response of CP55,940. When initially applied, ORG27569 does not appear to alter the inhibitory effect of CP55,940. Then, approximately 5 min after drug addition, the cAMP concentration in samples treated with ORG27569 diverge from the CP55,940-alone condition; increasing, and eventually rising above, the forskolin-alone condition. This phenomenon, and the analysis approach used to analyze such data, is described in detail in Cawston et al. (2013).

3. DESIGN AND VALIDATION OF V8-CAMYEL

While CAMYEL (Jiang et al., 2007) is excellent for use in large samples of easily transfectable cell lines, there are various experimental designs which would benefit from a biosensor with an increased bioluminescent output. Experiments on very small populations of cells, for example, are

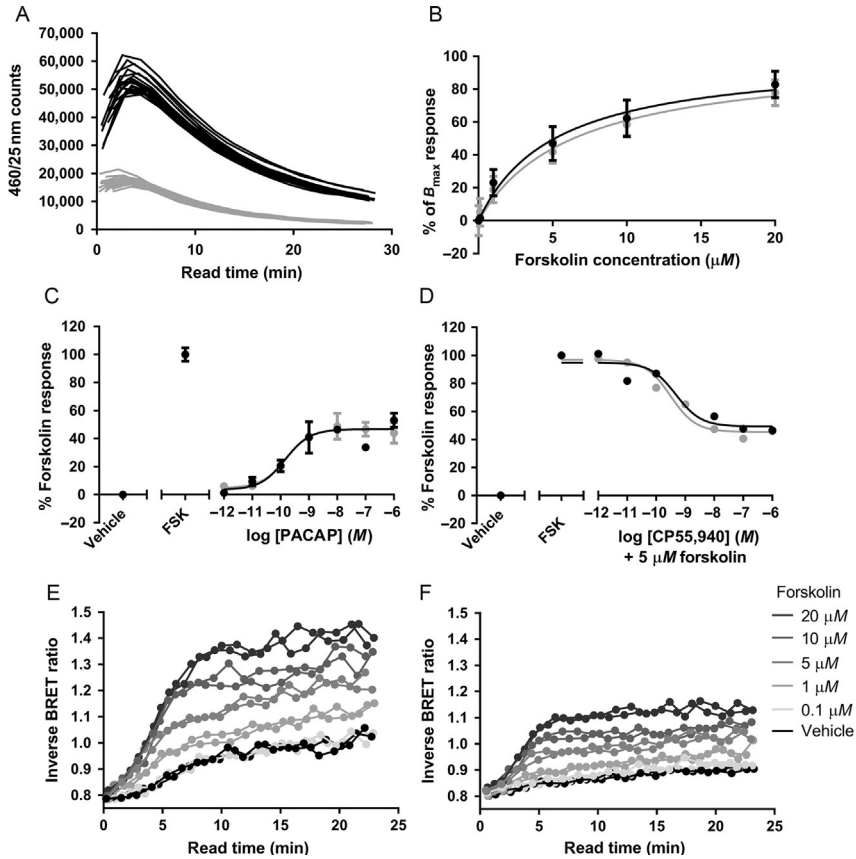

Fig. 2 Comparison of transiently transfected CAMYEL (*grey*) and V8-CAMYEL (*black*). (A) V8-CAMYEL has much higher light emission in the 460/25 nm channels, on average a 2.5-fold increase when transiently transfected ($P < 0.05$). (B–D) CAMYEL and V8-CAMYEL are similarly responsive to forskolin-, PACAP-, and CP-55,940-mediated changes to cellular cAMP. (E and F) Real-time responses of CAMYEL (E) and V8-CAMYEL (F) to various concentrations of forskolin.

technically challenging because there can be too few photons released from the biosensor per well to be efficiently detected by standard plate readers. In order to increase the bioluminescent output and increase stability in mammalian cytoplasm, the luciferase in CAMYEL was changed to the mutant Rluc8 (Loening, Fenn, Wu, & Gambhir, 2006) and the fluorescent acceptor was changed to Venus (Nagai et al., 2002). This pairing was predicted to increase the quantum yield of the biosensor. The new biosensor was called "V8-CAMYEL" (pcDNA3L-His-V8-CAMYEL) and can be obtained

from the corresponding author of this chapter. The modifications in V8-CAMYEL have allowed the measurement of biosensor activity from much smaller samples of cells in a conventional plate reader.

3.1 Validation of V8-CAMYEL

In order to validate V8-CAMYEL, it was tested alongside the original CAMYEL biosensor. These assays were designed to verify that modifications to the BRET pair did not alter the responsiveness of the biosensor to changes in cytoplasmic cAMP in mammalian cells. First, the biosensors were compared in their responsiveness to forskolin, an adenylate cyclase agonist, which directly modifies the production of cAMP. Then the biosensors were assayed for responsiveness to an endogenously expressed Gαs-coupled GPCR (PAC1, agonist: PACAP) and a stably transfected Gαi-coupled GPCR (CB1, agonist: CP55,940).

To determine that the V8-CAMYEL biosensor acted equivalently to the original CAMYEL construct, cAMP assays were performed using both biosensors. The HEK 3HA-hCB1 cell line was transiently transfected with either the CAMYEL or V8-CAMYEL construct and assayed as per the protocol for adherent cells described earlier.

The results of these assays are shown in Fig. 2. As predicted, V8-CAMYEL had much higher light emissions in both the 460/25 nm and 535/25 nm channels, on average a 2.5-fold increase when transiently transfected (Fig. 2A). AUC measurements were taken across 20 min and used to generate concentration–response curves for forskolin and CP55,940. In the case of PACAP, data were collected 5 min after agonist addition for 15 min. V8-CAMYEL showed equivalent sensitivity to these agonist-induced cAMP changes compared to the original CAMYEL biosensor.

The overall range of inverse BRET ratios measured is reduced in V8-CAMYEL compared to the parental construct, as shown in Fig. 2E and F. This indicates that, ratiometrically, Venus emissions in V8-CAMYEL are higher relative to luciferase than in the original construct, thus making the inverse BRET ratio lower. This is not entirely surprising, as the increased quantum yield of Rluc8 (Loening et al., 2006) may increase the energy transfer to Venus. There is also likely to be a higher proportion of functional Venus fluorophore in V8-CAMYEL, as this acceptor protein matures faster than other YFP derivatives (Nagai et al., 2002). However, when drug responses are normalized to an internal control (e.g., 5 μ*M* forskolin), V8-CAMYEL shows an improved signal-to-noise ratio, approximately 1.78 ± 0.03-fold higher than the original CAMYEL biosensor across the dynamic range.

Overall, V8-CAMYEL was found to perform equivalently to the original CAMYEL biosensor, but with the considerable advantage of increased total luminescence. This allows us to obtain a useable biosensor signal from extremely small samples of cells, using a standard luminescence plate reader.

3.1.1 Stable Cell Lines

Stable cells lines expressing V8-CAMYEL can be used to streamline experimental workflow. In this example, HEK293 FlpIn cells (Invitrogen, CA, USA) were stably transfected with CAMYEL or V8-CAMYEL using the standard, random-integration transfection method, in order to allow the FlpIn site to be used for future transfection. After selection, both cell lines contained very similar levels of biosensor expression. Here, we show example data from these cell lines assayed in the suspension assay format described above, using 384-well microtiter plates.

Fig. 3 shows that a stable cell line expressing V8-CAMYEL maintains a useable window for measuring cAMP signaling, even when diluted to very low cell numbers. Furthermore, when AUC analysis is an acceptable

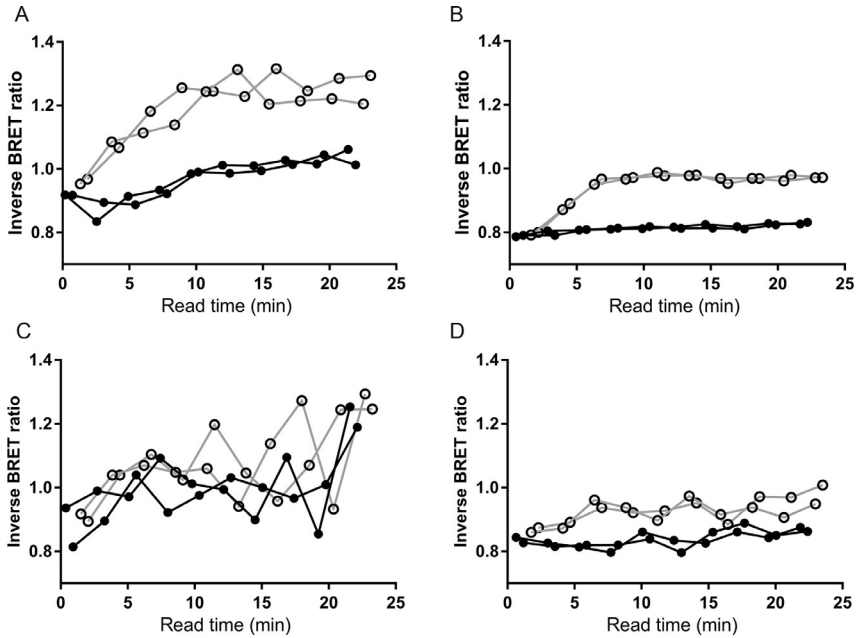

Fig. 3 HEK293 FlpIn cells were stably transfected with (A and C) CAMYEL or (B and D) V8-CAMYEL and treated with vehicle (*black*) and forskolin (*gray*). Cells were diluted to (A and B) 20,000 cells/well or (C and D) 1250 cells/well in a 384-well plate. Duplicate wells of cells treated at the same time are shown for each condition, plotted as non-normalized inverse BRET ratios.

measurement (thus averaging out some assay noise), HEK293 FlpIn V8-CAMYEL cells can be diluted much further; we have found these cells can be diluted to 312 cells/well. This opens the possibility of using this biosensor with hard to transfect cells, or primary cells where cells are less abundant.

4. SUMMARY

Transfectable cAMP biosensors have proven to be an invaluable tool in the analysis of cannabinoid receptor signaling. These biosensors allow for measurement of cellular cAMP in real time, and permit the detection of subtle and transient changes in ligand-induced signaling. Here, we describe the use of BRET-based biosensors, which are particularly suited to moderate- and high-throughput assays, such as drug screening. These assay systems can be modified to measure many different variables, for example, by altering the order of drug additions. The only requirement of a BRET-based biosensor is that the luciferase substrate is added before measurement begins. Using an extra-bright BRET biosensor, such as V8-CAMYEL, or stably transfecting cells with biosensor, allows for a more robust assay protocol. These revisions permit further assay miniaturization, and reduce assay preparation time and interexperimental variation.

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