Australian Government
Department of Defence
Science and Technology

Measurement of Intracellular cAMP using a BRET Biosensor

Georgina Thompson

Land Division
Defence Science and Technology Group

DST-Group-TN-1795

ABSTRACT

G protein-coupled receptors (GPCRs) are the targets of some chemical warfare agents, and many medical countermeasures to these agents. Techniques for measuring GPCR-mediated cell signalling are essential for chemical warfare agent research and medical countermeasure development. However, commercially available kits for measuring GPCR signalling are typically very expensive. This report describes a simple cost-effective method of measuring the activity of GPCRs coupled to the cAMP signalling pathway. The assay utilises a commonly used cAMP bioluminescence resonance energy transfer (BRET) biosensor to detect changes in cAMP levels in live cells. This method was used to measure activation, antagonism and allosteric modulation of a muscarinic acetylcholine receptor in a cultured mammalian cell line.

Produced by

Land Division
Defence Science and Technology Group
506 Lorimer Street
Fishermans Bend, VIC 3207

Telephone: 1300 333 362

July 2018
AR-017-238

APPROVED FOR PUBLIC RELEASE

Measurement of intracellular cAMP using a BRET biosensor

Executive Summary

The Agent-based Genomics and Cell Biology Science and Technology Capability (AG&CB STC) is developing a capability to investigate the *in vitro* pharmacology of G protein coupled receptors (GPCRs). GPCRs are the target of 35-40% of currently available pharmaceuticals and are major targets for new drug development. These receptors are also the target of some traditional chemical warfare agents, many emerging threat agents and their medical countermeasures (MCMs). Thus, a specific focus on GPCR pharmacology will enable the AG&CB STC to contribute to the collaborative development of new MCM drugs as well as the biological hazard assessment of chemical agents.

Methods to quantitatively measure the activation or inhibition of GPCRs by compounds of interest are essential for the AG&CB STC's *in vitro* pharmacology program. Many GPCRs, including opioid, muscarinic, dopaminergic and adrenergic receptors, are coupled to the cyclic adenosine monophosphate (cAMP) signalling pathway. A simple cost-effective method for *in vitro* measurement of intracellular cAMP levels in live cultured cells involves transfection of cells to express a bioluminescence resonance energy transfer (BRET) biosensor for cAMP.

This technical note describes the successful implementation of this method to measure cAMP levels in a mammalian cell line stably overexpressing muscarinic acetylcholine receptor 2 (M2). Stimulation, antagonism and allosteric modulation of the M2 receptor are demonstrated.

This method will be a core technique in the suite of *in vitro* pharmacology assays for the AG&CB STC and will be applied to both the hazard assessment of chemical agents as well as the assessment of novel MCMs currently under development. For a more comprehensive analysis of compounds of interest, this method can be easily adapted for the measurement of other GPCR signalling pathways using BRET-based biosensors described in the open literature.

This page is intentionally blank.

Contents

This page is intentionally blank.

Glossary

2-PAM	2-pyridine aldoxime methyl chloride
AC	adenylyl cyclase
ACh	acetylcholine
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
CAMYEL	cAMP sensor using YFP-Epac-RLuc
CHO	Chinese Hamster Ovary
DMEM	Dulbecco's Modified Eagle Medium
EC_{xx}	XX% of maximal effective concentration
EPAC	exchange protein directly activated by cAMP
FBS	foetal bovine serum
Fsk	forskolin
GPCR	G Protein Coupled Receptor
HBSS	Hank's balanced salt solution
M2	muscarinic acetylcholine receptor 2
PEI	polyethylenemine
RLuc	*Renilla* luciferase
SEM	standard error of the mean
YFP	Yellow Fluorescent Protein

This page is intentionally blank.

1. Introduction

G protein-coupled receptors (GPCRs) are cell surface receptors that are the targets of traditional and non-traditional chemical warfare agents, and many of the medical countermeasures to these agents. Therefore, methods of quantifying GPCR-mediated signalling are necessary for the assessment of existing and new antidote compounds, and the hazard assessment of emerging threat agents.

The signalling pathways activated by a GPCR depend on which G protein subtype the receptor couples to on the intracellular domain of the receptor. The predominant signalling pathway for Gs- and Gi-coupled receptors is adenylyl cyclase (AC) activation and inhibition, respectively [1]. AC activation stimulates production of the second messenger molecule cyclic adenosine monophosphate (cAMP) whereas AC inhibition leads to a decrease in intracellular cAMP.

There are numerous commercially available kits for measuring cAMP in cultured cells; however, they are typically very expensive, time-consuming and cannot be used in live cell cultures. Alternatively, bioluminescence resonance energy transfer (BRET) assays are a relatively simple, rapid and cost effective method of measuring cAMP levels in real time in live cultured cells. BRET is a naturally occurring phenomenon where energy from a donor luminescence enzyme is transferred to a complementary acceptor fluorophore. The excitation of the fluorophore only occurs if the donor enzyme is in close proximity, allowing BRET to be used to measure protein-protein interactions in live cells [2]. BRET-based bioassays can be used to measure activation of several GPCR signalling pathways by expression of specially designed BRET sensors [3, 4]. cAMP levels in live cultured cells can be measured using a cAMP BRET sensor that is comprised of a cAMP binding protein, EPAC, flanked by a BRET pair: *Renilla* luciferase (RLuc) and yellow fluorescent protein (YFP) [5]. The sensor, designated 'CAMYEL' (cAMP sensor using YFP-Epac-RLuc), changes conformation in response to increasing levels of cAMP, resulting in a loss of BRET intensity (Figure 1).

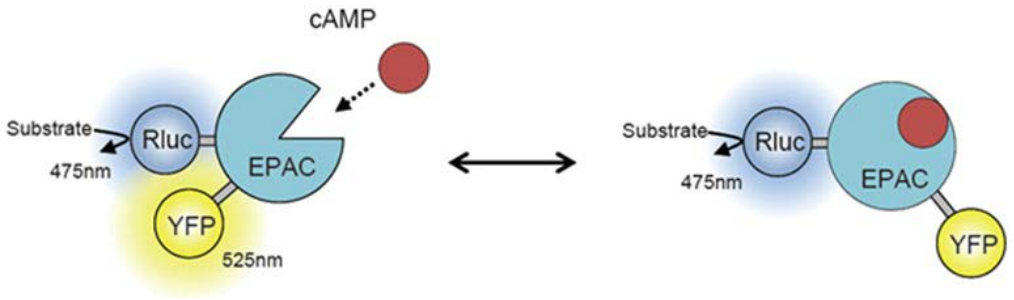

Figure 1 *CAMYEL BRET biosensor. CAMYEL (cAMP sensor using YFP-Epac-RLuc) changes conformation in response to increasing levels of cAMP, resulting in a loss of BRET intensity.*

This technical note describes the successful use of this method to measure inhibition of intracellular cAMP production in a Chinese Hamster Ovary (CHO) line stably overexpressing the Gi-coupled human muscarinic acetylcholine receptor 2 (M2) following activation of by acetylcholine (ACh) [6]. This method can be easily adapted to measure activation of other signalling pathways using BRET constructs to measure β-arrestin recruitment, receptor trafficking, G protein activation and more [7, 8].

2. Methods

2.1. Materials

Polyethylenemine (PEI) was purchased from Bioscientific (Gymea, NSW, Australia). Coelenterazine-h was purchased from Nanolight Technologies (Pinetop, AZ, USA). A DNA mammalian expression plasmid encoding CAMYEL was purchased from ATCC (Manassas, VA, USA; catalogue # MBA-277). Hygromycin B, Hanks balanced salt solution (HBSS) and all other routine cell culture reagents were obtained from Life Technologies (Mulgrave, VIC, Australia). All other chemicals were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). FlpIn-CHO-M2 cells were a gift from Arthur Christopoulos (Monash Institute of Pharmaceutical Sciences, Australia).

2.2. Cell Transfection

CHO cells were maintained and cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) containing 5% foetal bovine serum (FBS) and 600µg/ml hygromycin B at 37°C under a humidified atmosphere containing 5% CO_2. Approximately 10^6 FlpIn-CHO-M2 cells were grown overnight in 10cm dishes, then transient transfection of cells was performed using 6:1 ratio of PEI (12µg) to CAMYEL DNA (2µg). After 24h, cells were detached with trypsin and transferred to solid white TC-treated 96-well plates (Corning, USA).

2.3. cAMP Assay

The ability of ligands to inhibit forskolin-induced cAMP production was assessed in FlpIn-CHO-M2 cells transiently transfected to express the CAMYEL cAMP BRET biosensor. 48h after transfection, cells were rinsed and pre-incubated in HBSS for 30 min at 37°C. Cells were then incubated with the Rluc substrate coelenterazine-h, final concentration 5µM, for 5 min, followed by a further 5 min incubation with varying concentrations of ACh, either alone or in combination with antagonists or modulators. Forskolin was then added to a final concentration of 10µM. After 5 min, the YFP and the Rluc emissions were measured using a PolarSTAR Omega microplate reader (BMG Labtech, Ortenberg, Germany) that allows for sequential integration of the signals detected at 475 ± 30 and 535 ± 30 nm, using filters with the appropriate band pass. Data are presented as a BRET ratio, calculated as

the ratio of YFP to Rluc signals, and expressed as the percentage of the forskolin-induced signal in the absence of agonist (where 0% represents the baseline cAMP level in the absence of forskolin and agonist). Data analysis and graphing were preformed using Prism 6 software from GraphPad (La Jolla, CA, USA).

3. Results

3.1. Assay Optimisation

Since different cell lines express different levels of adenylyl cyclases, the optimal concentration of forskolin required for the assay needs to be determined for each cell line. A concentration-response experiment for forskolin (10µM – 10nM) without agonist was performed to determine the EC_{80} of forskolin-induced cAMP production in FlpIn-CHO-M2 cells. cAMP levels were measured after 5minof stimulation with forskolin (Figure 2). A $log_{10}EC_{80}$ of -6.04M was determined and, as a result, all subsequent experiments were performed using log_{10}-6.0M forskolin (final concentration in assay).

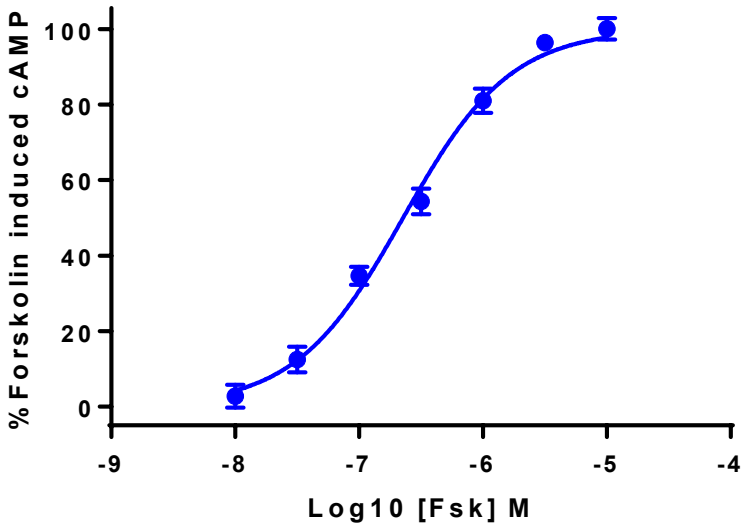

Figure 2 *Concentration-response curve of forskolin in FlpIn-CHO-M2 cells. Data expressed as mean±SEM, n=3. A $log_{10}EC_{80}$ = -6.04M was calculated using non-linear regression analysis and performed using Prism 6.*

3.2. Agonist-induced cAMP Inhibition

In order to measure agonist-induced inhibition of cAMP, a concentration-response experiment for the muscarinic agonist ACh (1µM – 100pM) was performed in FlpIn-CHO-M2 cells. As it is difficult to measure a decrease in already-low baseline levels of intracellular cAMP, the baseline level of cAMP was artificially increased by incubation with forskolin for 5min prior to the addition of ACh. cAMP levels were measured after 10min of stimulation with ACh (Figure 3). ACh stimulation resulted in a maximum cAMP inhibition (Emax) of 52.5% and $\log_{10}EC_{50}$ of -7.55 M.

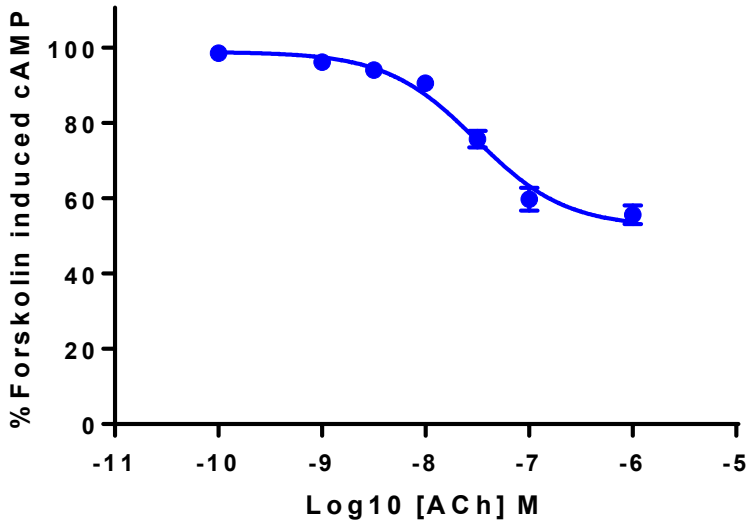

Figure 3 *Concentration-response curve of ACh in FlpIn-CHO-M2 cells. Data expressed as mean±SEM, n=6. A $\log_{10}EC_{50}$ = -7.55M was calculated using non-linear regression analysis and performed using Prism 6.*

3.3. Antagonism and Allosteric Modulation

In order to measure antagonism and allosteric modulation of agonist-induced inhibition of cAMP, CAMYEL expressing cells were pre-incubated for 30min with 1µM atropine, 2-PAM or vehicle. Subsequently, a concentration-response experiment for ACh (1µM – 100pM) was performed in FlpIn-CHO-M2 cells, as previously described (Figure 4). Pre-incubation with atropine resulted in complete antagonism of ACh-induced cAMP inhibition, whereas pre-incubation with 2-PAM resulted in a reduction in Emax (68.2%) and a small reduction in $\log_{10}EC_{50}$ (-7.01 M), consistent with negative modulation.

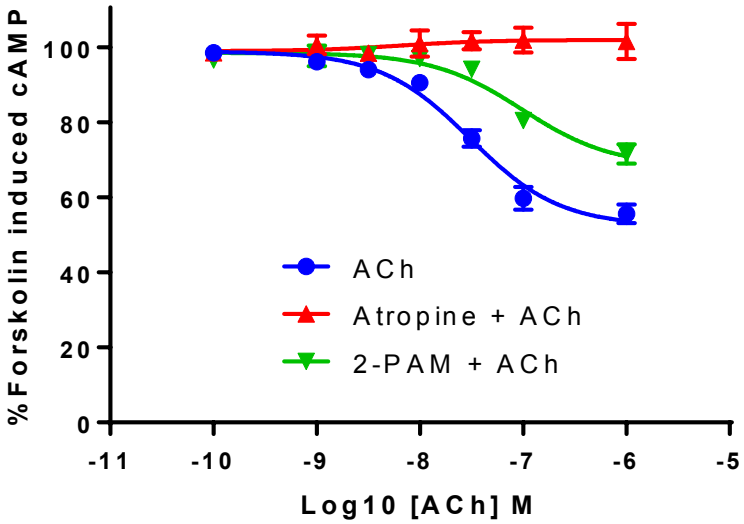

Figure 4 *Concentration-response curve of ACh with atropine or 2-PAM in FlpIn-CHO-M2 cells. Data expressed as mean±SEM, n=4. Atropine completely antagonised ACh-induced cAMP inhibition, whereas 2-PAM negatively modulated the activity of ACh ($log_{10}EC_{50}$ -7.01 M).*

4. References

1. Ejendal, K.F.K., et al., *Adenylyl cyclase isoform-specific signaling of GPCRs*, in *G Protein-Coupled Receptors: Structure, Signaling, and Physiology*. 2010, Cambridge University Press. p. 189-216.

2. Boute, N., et al., *The use of resonance energy transfer in high-throughput screening: BRET versus FRET.* Trends Pharmacol Sci, 2002. **23**(8): p. 351-4.

3. Matthiesen, K. and Nielsen, J., *Cyclic AMP control measured in two compartments in HEK293 cells: phosphodiesterase K(M) is more important than phosphodiesterase localization.* PLoS One, 2011. **6**(9): p. e24392.

4. Percherancier, Y., et al., *Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers.* J Biol Chem, 2005. **280**(11): p. 9895-903.

5. Jiang, L.I., et al., *Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway.* J Biol Chem, 2007. **282**(14): p. 10576-84.

6. Kruse, A.C., et al., *Muscarinic acetylcholine receptors: novel opportunities for drug development.* Nat Rev Drug Discov, 2014. **13**(7): p. 549-60.

7. Arts, R., et al., *Engineering BRET-Sensor Proteins.* Methods Enzymol, 2017. **589**: p. 87-114.

8. Sleno, R., et al., *Designing BRET-based conformational biosensors for G protein-coupled receptors.* Methods, 2016. **92**: p. 11-8.

Appendix A cAMP BRET Assay: Bench Protocol

Materials

- 150 mM NaCl - filter sterilised
- 1µg/µl Polyethylenemine (PEI) (Polysciences: Linear, MW 25,000 Cat No: 23966)

  Dissolve 1mg/ml in 150mM NaCl at pH7.0 on stirrer with gentle heating. Aliquot and store at -20 ^{o}C. Working stocks can be stored at 4 ^{o}C for 1 week.

- Hanks Balanced Salt solution (HBSS)

  0.34mM Na_2HPO_4, 4.16mM $NaHCO_3$, 0.44mM KH_2PO_4, 138mM NaCl, 0.40mM $MgSO_4$, 0.49mM $MgCl_2$, 1.26mM $CaCl_2$, 5.33mM KCl, 5.56mM D-glucose pH7.4

- 5mM Coelenterazine h (Nanolight Technologies 1 mg, Cat No: 301-1)

  Dissolve a 500µg vial in 246µl 100% ethanol, store at -20 ^{o}C for 1 month, protect from light.

- 10mM Forskolin in DMSO, store at -20 ^{o}C – determine EC_{90} for the cell line

Method

Day 1: Seed 1-1.5x10^6 cells/10cm dish

Day 2: Transfection:

1. DNA dilution: 2ug CAMYEL in 250µl of NaCl.
2. PEI dilution (1:6 DNA:PEI): 12µl PEI in 250 µl of NaCl.
3. Add the PEI solution to the DNA.
4. Vortex immediately for 3sec and incubate for 10min at RT. In the meantime, replace medium in the dish with fresh medium (10ml).
5. Add PEI/DNA complex to the cells (drop wise) (500µl/plate)

Day 3: Trypsinise cells and dilute up to a total of 10-20 ml with growth medium. Add 100µl/well to white 96-well plates (to achieve ~80-90% confluency). Add cells to a few wells of a clear plate to check on the health and confluency of the cells.

Day 4: Assay – Perform 48hours after transfection to achieve optimal expression.

1. Aspirate medium and wash once with 100µl/well HBSS @ 37˚C
2. Add 70µl (Gi) or 80µl (Gs) HBSS/well and incubate at least 30min @37˚C
   (Add 60µl (Gi) or 70µl (Gs) for antagonist/modulator experiments)
3. Prepare drug solutions and forskolin at 10x final concentration in HBSS
4. Optional: Add 10µl/well antagonist/modulator and pre-incubate for 30min
5. Prepare 1ml/plate of 50µM Coelenterazine h in HBSS
   (i.e. 1 in 100 from stock)
6. Add 10µl/well Coelenterazine h and incubate for 5 min @ 37˚C
7. Add 10µl/well agonist and incubate for 5min @ 37˚C
   (10min for Gs - skip step 8)
8. Gi only: Add 10µl/well forskolin and incubate for 5min @ 37˚C
9. Measure plate using the luminescence optic on the Polarstar.
   Select luminescence protocol BRET1 (Simultaneous dual emission: 475-30/535-30nm with 1s measurement interval).

DEFENCE SCIENCE AND TECHNOLOGY GROUP DOCUMENT CONTROL DATA			1. DLM/CAVEAT (OF DOCUMENT) None
2. TITLE Measurement of intracellular cAMP using a BRET biosensor		3. SECURITY CLASSIFICATION (FOR UNCLASSIFIED LIMITED RELEASE USE (U/L) NEXT TO DOCUMENT CLASSIFICATION) Document (U) Title (U) Abstract (U)	
4. AUTHOR(S) Georgina Thompson		5. CORPORATE AUTHOR Defence Science and Technology Group 506 Lorimer Street Fishermans Bend, VIC 3207	
6a. DST GROUP NUMBER DST-Group-TN-1795	6b. AR NUMBER AR-017-238	6c. TYPE OF REPORT Technical Note	7. DOCUMENT DATE July 2018
8. OBJECTIVE ID	9.TASK NUMBER N/A	10.TASK SPONSOR N/A	
11. MSTC Chemical and Biological Defence		12. STC Agent-based Genomics and Cell Biology	
13. DOWNGRADING/DELIMITING INSTRUCTIONS		14. RELEASE AUTHORITY Chief, Land Division	

15. SECONDARY RELEASE STATEMENT OF THIS DOCUMENT

Approved for public release.

OVERSEAS ENQUIRIES OUTSIDE STATED LIMITATIONS SHOULD BE REFERRED THROUGH DOCUMENT EXCHANGE, PO BOX 1500, EDINBURGH, SA 5111

16. DELIBERATE ANNOUNCEMENT

Australian Department of Defence and Defence Force Personnel.

17. CITATION IN OTHER DOCUMENTS

Yes

18. RESEARCH LIBRARY THESAURUS

Pharmacology, Biochemistry, Drugs, Chemical weapons, Chemical hazards

19. ABSTRACT

G protein-coupled receptors (GPCRs) are the targets of some chemical warfare agents, and many medical countermeasures to these agents. Techniques for measuring GPCR-mediated cell signalling are essential for chemical warfare agent research and medical countermeasure development. However, commercially available kits for measuring GPCR signalling are typically very expensive. This report describes a simple cost-effective method of measuring the activity of GPCRs coupled to the cAMP signalling pathway. The assay utilises a commonly used cAMP bioluminescence resonance energy transfer (BRET) biosensor to detect changes in cAMP levels in live cells. This method was used to measure activation, antagonism and allosteric modulation of a muscarinic acetylcholine receptor in a cultured mammalian cell line.