Differential activation of G proteins by synthetic cannabinoid receptor agonists, utilizing the CAMYEL BRET biosensor

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- **Synthetic cannabinoid receptor agonists can activate both $G\alpha_{i/o}$ and $G\alpha_s$ protein pathways via CB1 GPCRs**
- **The CAMYEL BRET biosensor allows real-time monitoring of cAMP levels in live cells**
- **With the simultaneous dual emission feature, the PHERAstar provides high speed and outstanding sensitivity**

Introduction

Cannabinoid Receptor 1 (CB1) agonists

Synthetic cannabinoid receptor agonists (SCRAs) have been extensively used as tools to study the two cannabinoid G protein-coupled receptors (GPCR's), CB1 and CB2. Unfortunately, SCRAs now represent the most rapidly growing class of new psychoactive substances associated with drug misuse. SCRAs pose serious health risks with adverse effects such as severe vomiting, chest pain, increased heart rate, kidney damage, seizures/unconsciousness and even death. The molecular mechanisms through which SCRA's exert their toxic effects remains unclear.[1]

GPCR's in general, span the outer membrane of a cell and are coupled to a trimeric G protein complex (Gα, Gβ, Gγ) inside the cell. Upon agonist activation of the GPCR, the trimeric G protein complex dissociates releasing Ga (of which there are four major subtypes: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, $G\alpha_{12/13}$) and $G\beta_g$ proteins within the cell. Many cellular responses can be initiated through the Ga proteins, depending on which of the four major subtypes is present in a particular cell. Most relevant here is Gas which stimulates adenyl cyclase activity leading to an increase in cAMP, which in turn initiates further downstream responses. In contrast Gai, inhibits adenyl cyclase leading to a decrease in cAMP production.

Previous studies have shown that SCRAs are agonists at both CB1 and CB2 receptors, with the psychoactive effects attributed to CB1 activation. It is also known that cannabinoid receptor G protein signaling for SCRAs is predominantly through the $G\alpha_i$ protein family.

In this study[2], a representative group of sixteen cannabinoids was chosen (twelve confirmed in patients admitted to emergency departments with SCRA toxicity and 4 reference compounds) and determined whether these cannabinoids signal via the Gi and/or Gs pathways, using a Bioluminescence Resonance Energy Transfer (BRET) biosensor.

Assay Principle

The CAYMEL BRET biosensor[3] is composed of the EPAC protein bound to both a luciferase (RLuc) and yellow fluorescent protein (YFP) as shown in fig. 1. In the absence of cAMP, the conformation of EPAC allows Rluc and YFP to be in close proximity. A BRET signal can therefore occur by addition of Coelenterazine (RLuc substrate). As cAMP is formed in the cell, it binds EPAC, which in turn causes a conformational change that diminishes the BRET signal as the concentration of cAMP increases.

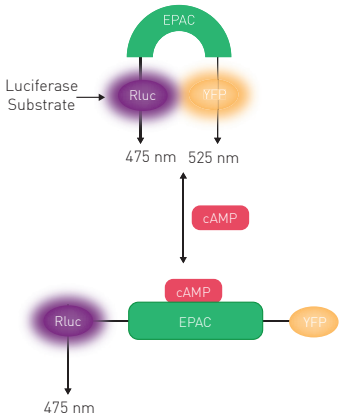

Fig. 1: Assay Principle: CAMYEL BRET biosensor. CAMYEL (cAMP sensor using YFP-Epac-RLuc) changes conformation in response to increasing levels of cAMP, resulting in a loss of BRET intensity.

Materials & Methods

- 96-well microplate, white, clear bottom, poly D lysine coated (Sigma-Aldrich)
- PHERAstar *FS*, BRET 1 optic module
- HEK293 Flpln cells stably expressing CB1
- pcDNA3L-His-CAMYEL plasmid

Transfection Procedure

Human HEK293 cells, stably expressing CB1 receptor (HEK-CB1)[2] were transfected with pcDNA3L-His-CAMYEL (5ug) plasmid using the linear polyethylenimine (PEI) protocol. The PEI/DNA complex mixture was sequentially added in a 1:6 ratio and cells incubated at 37 °C and 5% CO_2 for 24h.

GPCR activation and cAMP measurement

To determine whether the tested cannabinoids signal via $G\alpha_s$ and $G\alpha_i$ pathways, transfected cells stably expressing CB1 and CAMYEL were treated/not treated with Pertussis Toxin (PTX). PTX inactivates the $G\alpha_i$ signaling pathway but has no effect on $G\alpha_s$ proteins. With PTX mediated inactivation of $G\alpha_i$ signaling, the sole effect of potential agonists on $G\alpha_s$ can be investigated. Both populations of cells were plated to a density of 100,000 cells per well. On the day of the assay, Forskolin was prepared (3µM final concentration). It is used to raise levels of cAMP so inhibition can be measured. Luciferase substrate (Coelenterazine H, 5µM) was added and the response to the subsequently added cannabinoid was measured at a final concentration of 10uM. CAMP levels were monitored in real time and in live cells using the CAYMEL BRET biosensor. Changes in the BRET ratio were measured with a PHERAstar plate reader at 37°C in kinetic mode, every 40 s for 20 min.

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Instrument settings

Optic settings	Luminescence, BRET 1 optic module, simultaneous dual emission	
	Em Filters	475-30, 535-30
General settings	Settling time	0.5 s
Kinetic settings	Number of cycles	30
	Cycle time	40 s
Incubation	37°C	

Results & Discussion

We initially measured CB1-mediated activation of $G\alpha_s$ proteins by different cannabinoids (PTX treated cells). Typical signal curves are shown in fig. 2A.

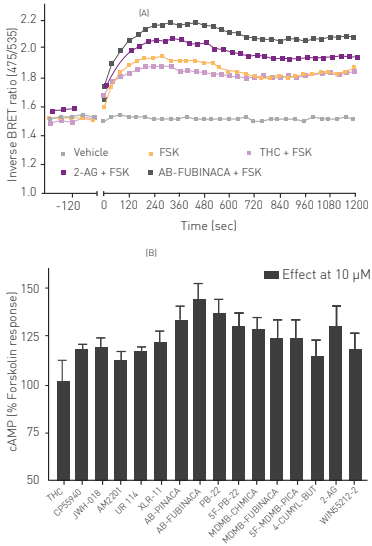

Fig. 2: **A)** Real-time measurement of stimulation of cAMP levels by 10µM THC, 2-arachidinoylglycerol and AB-FUBINACA in HEK-CB1 cells. **B)** Summary cAMP signaling peaks for 16 cannabinoids showing increase in cAMP levels above that of FSK (3µM) alone (100%)..

A reverse BRET ratio (475/535) was used so that an increase in ratio corresponded to an increase in cAMP. The area under the total signal curve was used for quantification. Sixteen cannabinoids were initially tested at a concentration of 10µM. It was found that all tested cannabinoids, increased cAMP levels (12-45%) above that produced by FSK alone (fig. 2B).
In contrast, THC did not significantly alter levels over FSK. Concentration response curves were then constructed for five structurally different compounds to determine EC_{50} and E_{max} values for activation of both the $G\alpha_s$ and $G\alpha_i$ signaling pathways. All the SCRAs tested activated CB1 via both pathways (tab.1) however all compounds were much more potent for $G\alpha_i$ as compared to $G\alpha_s$ pathways. The activation of CB1-$G\alpha_s$ by different cannabinoids showed a wide variation in E_{max} values and there was a significant difference in efficacy between the different compounds. The rank order of efficacy for stimulation via $G\alpha_s$ was: AB-FUBINACA ~PB22 >5F-MDMB-PICA> XLR-11>JWH-018. In contrast, all cannabinoids showed similar maximal activity at $G\alpha_i$.

Tab 1. EC_{50} and E_{max} values for selected agents

	G_i(-PTX)		G_s(+PTX)	
Cannabinoid	pEC_{50} (EC_{50} nM)	E_{max} (%FSK)	pEC_{50} (EC_{50} nM)	E_{max} (%FSK)
JWH-018	7.8 ± 0.2	64 ± 3	6.5 ± 0.7	114 ± 4
XLR-11	7.2 ± 0.2	63 ± 2	5.3 ± 0.8	124 ± 5
PB-22	8.6 ± 0.2	64 ± 3	7.2 ± 0.5	130 ± 3
AB-FUBINACA	9.0 ± 0.2	61 ± 2	6.4 ± 0.5	144 ± 12
5RF-MDMB_PICA	9.2 ± 0.2	60 ± 4	7.1 ± 0.4	126 ± 5

Conclusion

This study has shown that cannabinoid receptor agonists have significantly different maximal activities and potencies for the activation of CB1-mediated G protein stimulation and inhibition of FSK mediated cAMP signaling. It is hoped that these findings will provide a starting point to help predict the pharmacological characteristics of SCRAs that show differential activation of $G\alpha_i$ and $G\alpha_s$ coupling to CB1.
This study also establishes the eminent suitability of the PHERAstar for kinetic BRET assays. The PHERAstar combines both high sensitivity and speed (able to read two wavelengths simultaneously) making it ideal for high-throughput applications in all reading modes.

References

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