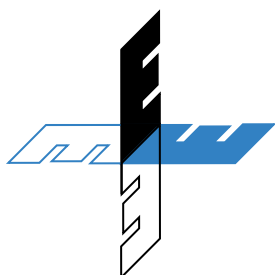


Homogeneous GTP γ S Assay for High Throughput Screening of GPCRs

Gregory Warner, Ph.D., Patricia Kasila[†],
and Harry Harney[†]*



Abstract

G Protein-Coupled Receptors (GPCRs) are a diverse family of membrane bound receptors that are involved in the transduction of extracellular messages from peptides, proteins, nucleotides, etc., into the cell. At least 6 families of GPCRs exist which show no sequence similarity. However, these receptors all control the activity of their respective enzymes, etc., via the catalysis of the GDP-GTP exchange on heterotrimeric G proteins ($G_{\alpha\beta\gamma}$).

This report demonstrates a homogeneous assay for measuring the binding of GTP to GPCR using the WGA FlashPlate® format. This assay allows for quantitative determination of agonist induced activation of [³⁵S]GTP γ S binding to isolated GPCR membranes without the need for the filtration and washing steps required in the most commonly used methodologies. The combination of the WGA FlashPlate format and this method allows for an easily adaptable high throughput screening assay for agonists of GPCRs.

Introduction

Wheat Germ Agglutinin (WGA) FlashPlates are a practical, easy-to-use, 96- or 384-well platform for high-throughput G Protein-Coupled Receptor (GPCR) binding assays. This technology incorporates proprietary covalent attachment of WGA to the microplate, providing a stable, high capacity binding capability. The interior of each FlashPlate well is permanently coated with a thin layer of polystyrene-based scintillant. The FlashPlate is then coated with proprietary proteins followed by Wheat Germ Agglutinin. The WGA captures receptors with carbohydrate moieties, thereby providing a platform for homogeneous, high-throughput receptor-ligand binding assays.

GPCR occupation by agonists leads to guanine nucleotide exchange. GDP bound to G_{α} , of the $G_{\alpha\beta\gamma}$ complex, dissociates and is replaced by GTP. G_{α} -GTP then detaches from the complex. Dissociation of the complex leads to $\beta\gamma$ and G_{α} -GTP subunits that are capable of downstream signaling. Activity of the subunits is controlled by GTPase activity that hydrolyzes the GTP to form G_{α} -GDP, which then associates with the $\beta\gamma$ subunits to reform the $G_{\alpha\beta\gamma}$ complex. The development of a radioactive, non-hydrolyzable analogue of GTP, [³⁵S]GTP γ S, led to the ability to measure agonist occupation of GPCR and distinguish between compounds for efficacy and activity. Unfortunately, the assay commonly performed requires filtration to remove unbound [³⁵S]GTP γ S prior to scintillation counting and is therefore limited in its applicability to high-throughput drug screening.

This report focuses on using the 96-well WGA FlashPlate format to provide a homogeneous assay for binding of [³⁵S]GTP γ S to GPCR, using the human mu and kappa opioid receptors as models, which may be applicable to high throughput screening for agonists of GPCR.

1

Experimental Procedures

Materials

- WGA FlashPlate PLUS, 96-well (PerkinElmer Life Sciences, Cat. # SMP105)
- Human Mu Opioid Receptor membranes (Receptor Biology, Cat # RB-HOM)
- Human Kappa Opioid Receptor membranes (Receptor Biology, Cat # RB-HOK)
- [³⁵S]GTPγS (1250 Ci/mmol, PerkinElmer Life Sciences, Cat. # NEG030H; 1050 Ci/mmol, Amersham, Cat. # SJ1320)
- [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO) (Sigma Chemical, Cat. #E-7384)
- U-69593 [(5α,7α,8β)-(+)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide] (Sigma Chemical, Cat. # U-103)

2

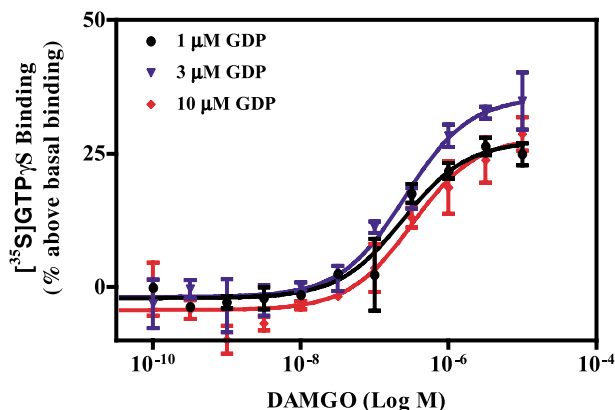
Experimental Procedures

Method

- Assay Buffer: 75 mM Tris, pH 7.5; 12.5 mM MgCl₂; 1 mM EDTA; 0.5% (w/v) BSA; 100 mM NaCl, 3 μM GDP in Distilled H₂O.
- Dilute human opioid receptor membranes in assay buffer to give 10 μg protein in 60 μl.
- Make 2X [³⁵S]GTPγS @ 0.8 nM in assay buffer.
- Mix assay buffer, receptor, [³⁵S]GTPγS, and agonist thoroughly and incubate 4 hr at RT.
- Bound radioactivity determined in MicroBeta®.

3

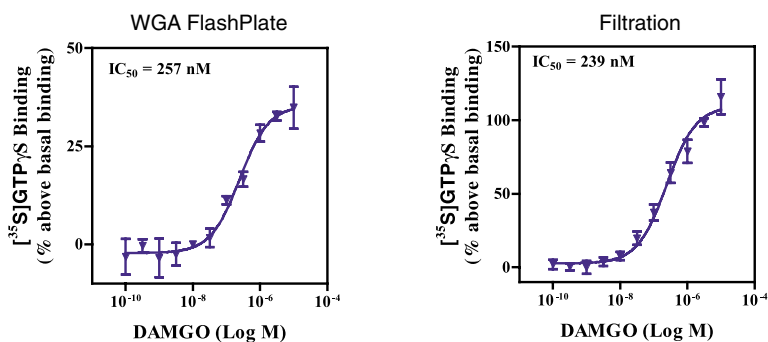
Effect of GDP Concentration on Agonist Induced Binding of GTP γ S to Human Mu Opioid Membranes in WGA FlashPlate PLUS



Human MOR membranes (10 μ g/well) added to 96-well WGA FlashPlate PLUS in the presence of either 1, 3, or 10 μ M GDP, 0.4 nM [35 S]GTP γ S, and increasing amounts of the mu opioid specific agonist, DAMGO. Plate was incubated at room temperature for 4 h prior to scintillation counting in MicroBeta.

4

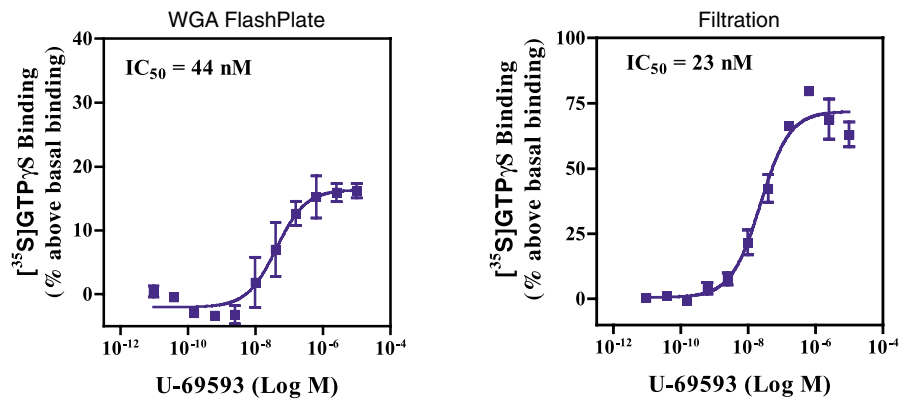
Comparison of WGA FlashPlate PLUS to Filtration in Agonist Induced Binding of GTP γ S to Human Mu Opioid Membranes



Human MOR membranes (10 μ g/well) added to either 96-well WGA FlashPlate PLUS or a low-protein binding assay plate in the presence of 3 μ M GDP, 0.4 nM [35 S]GTP γ S, and increasing amounts of the mu opioid specific agonist, DAMGO. Plates were incubated at room temperature for 2 h (low-protein binding assay plate) or 4 h (96-well WGA FlashPlate PLUS). Contents of low-protein binding assay plate were filtered through MultiScreen-FB filter plate prior to scintillation counting in MicroBeta.

5

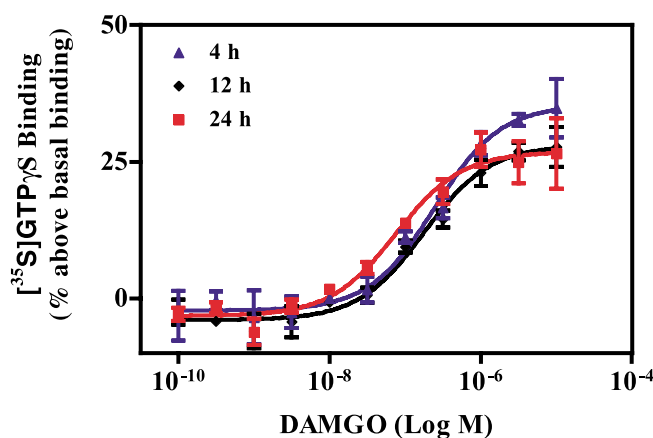
Agonist Induced Binding of GTP γ S to Human Kappa Opioid Membranes in WGA FlashPlate PLUS



Human KOR membranes (10 $\mu\text{g}/\text{well}$) added to either 96-well WGA FlashPlate PLUS or a low-protein binding assay plate in the presence of 3 μM GDP, 0.4 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, and increasing amounts of the kappa opioid specific agonist, U-69593. Plates were incubated at room temperature for 2 h (low-protein binding assay plate) or 4 h (96-well WGA FlashPlate PLUS). Contents of low-protein binding assay plate were filtered through MultiScreen-FB filter plate prior to scintillation counting in MicroBeta.

6

Kinetics of WGA FlashPlate PLUS Determination of GTP γ S Binding to Human Mu Opioid Membranes



Human MOR membranes (10 $\mu\text{g}/\text{well}$) added to 96-well WGA FlashPlate PLUS in the presence of 3 μM GDP, 0.4 nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, and increasing amounts of the mu opioid specific agonist, DAMGO. Plate was incubated at room temperature for up to 24 h prior to scintillation counting in MicroBeta.

Conclusion

- 96-well WGA FlashPlate PLUS can measure agonist induced increases in GTP γ S binding to GPCR membranes.
- Results from WGA FlashPlate PLUS and conventional filtration assay show comparable IC₅₀.
- Assay plate can be read as soon as 4 h or at least as long as 24 h after initiation without affecting results.
- Observed activation is somewhat less in WGA FlashPlate PLUS compared to filtration under the conditions used in this study.
- WGA FlashPlate PLUS assay is homogeneous, eliminates the need for filtration and washing, thus making it a method suitable for high throughput screening of GPCR agonists.

FlashPlate is a registered trademark of Packard Instrument Company, Inc. exclusively licensed to PerkinElmer Life Sciences, Inc. FlashPlate is protected under U.S. Patent 5,496,502 and foreign equivalents, to all of which PerkinElmer Life Sciences, Inc. holds an exclusive, worldwide license. MicroBeta is a registered trademark of PerkinElmer Life Sciences, Inc.



ENANTA Pharmaceuticals, Inc.



Worldwide Headquarters: PerkinElmer Life Sciences, 549 Albany Street, Boston, MA 02118-2512 USA (800) 551-2121

European Headquarters: PerkinElmer Life Sciences, Imperiastraat 8, B-1930 Zaventem Belgium +32 2 717 7911

H78480

© 2001 PerkinElmer Life Sciences, Inc.

www.perkinelmer.com/lifesciences