Chapter 10

Assay of GTPγS Binding in Autoradiography

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Abstract

Autoradiography of radiolabeled GTPγS ([^{35}S]GTPγS) binding is a relevant method to study the function of G protein-coupled receptors (GPCRs), in tissue sections. Here, we describe the protocol for such a binding autoradiography, suitable to investigate the functionality of CB_1 receptor in tissue slices from rodent brain.

Key words [^{35}S]GTPγS, Binding, Autoradiography, CB_1 receptor, G-proteins, Brain section

1 Introduction

[^{35}S]GTPγS binding autoradiography is a technique used to provide functional information on G protein-coupled receptors (GPCRs). It has been developed to measure the level of G protein activation following agonist occupation of a GPCR. This can be obtained by labeling receptor-coupled G proteins with a non-hydrolyzable radiolabeled analogue of GTP (GTPγS), in the presence of excess GDP [1].

GPCRs represent the largest family of membrane proteins and mediate many cellular processes [2]. Indeed, members of this family include receptors for a great variety of ligands (e.g., hormones, neurotransmitters, lipids, and even for direct sensory stimuli) [3]. The main endocannabinoid receptors, CB_1 and CB_2, belong to the GPCR family.

Structurally, GPCRs consist of an extracellular N terminus, seven transmembrane domains, and an intracellular C terminus. They couple with G proteins that are membrane-anchored heterotrimeric protein molecules responsible for signal transduction and amplification. G proteins are composed by α (45 kDa), β (35 kDa), and γ (8 kDa) subunits. The α and γ subunits are attached to the membrane by lipid anchors, and the former subunit binds either GDP or GTP, depending on whether the protein is inactive (GDP) or active (GTP). In the

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inactive state, the entire G protein-GDP complex binds a GPCR. Upon ligand binding, a conformational change of the GPCR activates the G protein, and GDP bound to the α subunit is physically replaced by GTP. At this point, the G protein subunits dissociate into the GTP-bound α subunit and a β-γ dimer. Both parts remain anchored to the membrane, but not to the GPCR, and can interact with several effector systems. Then, the GTPase activity of Gα subunit hydrolyzes GTP into GDP, allowing the α subunit to reassociate with the β-γ complex, and resetting the G protein to the inactive state [4].

The concept that receptor–G-protein complexes are still able to function in tissue sections came from Zarbin and colleagues in 1983 [5]. In 1986, the first autoradiographic visualization of guanine—nucleotide-binding proteins was obtained using [^{3}H]Gpp(NH)p in a rat brain section [6]. Then, in 1995 for the first time Sim and colleagues performed the [^{35}S]GTPγS binding autoradiography in rat brain cryosections after CB_1 receptor stimulation [7].

2 Materials

Prepare all stock solutions during the days preceding the assay, and store under appropriate temperature conditions.

2.1 Slide Preparation

1. Microscope slides (26×76 mm).
2. Gelatine: Dissolve 2.5 g of gelatine in 0.5 l of heated, deionized water (temperature should not exceed 60 °C). After the gelatine has dissolved, let cool the solution at room temperature. Add 0.25 g of chromium potassium sulfated. Mix and filter solution before use. Store at 4 °C.
3. Racks.

2.2 Tissue Preparation

1. Cryostat (Leica, Biosystem, Nussloch GmbH, Germany).

2.3 Assay Components

1. Liquid-repellent slide marker pen (PAP-PEN).
2. 1 M Tris–HCl, pH 7.4: Weight 157.6 g Tris hydrochloride and transfer to a glass beaker. Add water to a volume of 800 ml. Mix and adjust pH with 10 M NaOH. Make up to 1 l with water. Store at 4 °C (*see* **Note 1**).
3. 60 mM $MgCl_2$ stock solution: Add 600 µl of solution in 10 ml of water. Store at 4 °C.
4. 0.25 M EGTA stock solution: Dissolve 0.951 g in 10 ml of water. Store at 4 °C.
5. Bovine serum albumin (BSA): 0.5 % (wt/vol) in assay buffer. Lyophilized powder, essentially fatty acid free ≥96 %. Store at 4 °C.

6. 1.6 M NaCl stock solution: Dissolve 4.68 g in 50 ml of water. Store at 4 °C.
7. 1000 U Adenosine deaminase stock solution: Add 1 µl of adenosine deaminase in 999 µl of water, to prepare (fresh each time) a working solution.
8. Assay buffer: 1 M Tris–HCl, pH 7.4, 0.06 M $MgCl_2$, 0.25 M EGTA, 1.6 M NaCl, 0.5 % BSA, 1000 U adenosine deaminase. Mix all components and make up to a convenient volume with water at room temperature (*see* **Note 2**).
9. 300 mM Guanosine 5′-diphosphate disodium salt (GDP) stock solution: Dissolve 146 mg in 1 ml of assay buffer without 0.5 % BSA. Store at −20 °C.
10. Guanosine 5′-[^{35}S]triphosphate triethylammonium salt ([^{35}S]GTPγS, 1250 Ci/mmol in 250 µl): Add 2 µl of [^{35}S]GTPγS in 498 µl of 50 mM Tris–HCl, to prepare a 40 nM [^{35}S]GTPγS stock solution. Add 50 µl of 40 nM stock solution in 450 µl of 50 mM Tris–HCl, to prepare the working solution. Store in a safe location at −20 °C (*see* **Note 3**).
11. 1 mM CP-55,940 stock solution: Dissolve 3.86 mg in 10 ml of ethanol. Store at −20 °C.
12. Carestream® Kodak® BioMax® MR film (18 cm × 24 cm): Store at room temperature.
13. GE Healthcare Amersham™ Hypercassette™ Autoradiography Cassette.
14. Developer D-19 and fixer Kodak™ Processing chemicals: Developer: Dissolve 607 g in 3.8 l of hot water (38–52 °C), and stir until complete dissolution. Fixer: Dissolve 700 g in 3.8 l of water (18–25 °C), and stir until complete dissolution (*see* **Note 4**). It is also possible to use a developing machine.

3 Methods

3.1 Gelatine-Coated Slides

1. Place the slides into racks.
2. Dip the racks containing the slides three times (30 s each) into the gelatin solution.
3. Remove the excess solution from the racks. For better drainage, gently tap the racks against a piece of paper (*see* **Note 5**).
4. Cover each rack with an aluminum foil and dry overnight at 50 °C.
5. Put the dried slides into the boxes to protect from dust. Enumerate them before use.

3.2 Tissue Preparation

1. Rat or mouse brain is rapidly removed, frozen in liquid nitrogen, and stored at −80 °C until sectioning.
2. Cut 20 μm thick brain sections according to the rat or mouse brain atlas (e.g., Paxinos and Watson), at −20 °C using the cryostat. For each brain area of interest (according to the atlas coordinates), prepare a set of slides. Proceed collecting the first section for each slide, and then repeat the procedure collecting the second section for each slide. Each slide can contain till four rat brain sections or five mouse brain sections. In this way, each slide contains the area of interest in its whole extension and is comparable to the others (*see* **Note 6**).
3. Thaw-mount the sections onto gelatin-coated microscope slides and store at −80 °C until use (*see* **Note 7**).

3.3 Assay

The assay is composed of three consecutive incubations. All procedures must be carried out at room temperature (25 °C) and attention must be paid when handling and storing of radioisotopes. These must be used in the dedicated rooms.

1. Lead the slides at room temperature to thaw. For the binding assay two consecutive slides are needed (e.g., even and odd number) for each brain area of interest.
2. When the slides are dried, surround the sections with the liquid blocker PAP-PEN (*see* **Note 8**).
3. Prepare an appropriate volume of the assay buffer (400 μl per slide × 3 incubations) by mixing 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.5 % BSA, 10 mU/ml adenosine deaminase, and deionized water in a graduate cylinder with the aid of a magnetic stirrer (*see* **Note 9**).
4. Preincubate slides in assay buffer for 10 min at room temperature by adding 400 μl of assay buffer and ensure that all the sections are covered.
5. Add 300 mM GDP to the remaining assay buffer to reach a final concentration of 3 mM GDP (1:100 dilution) (*see* **Note 10**).
6. Drain the slides and re-incubate in assay buffer supplemented with 3 mM GDP at room temperature for 15 min.
7. Under proper safe conditions, lead the working solution of [^{35}S]GTPγS at room temperature to thaw. Dilute the [^{35}S]GTPγS 1:100 in assay buffer containing 3 mM GDP. Add an appropriate volume of 4 nM [^{35}S]GTPγS in the assay buffer, and split equal volumes of the solution in two conical tubes. Add 1 mM CP-55,940 to one tube to reach a final concentration of 5 μM CP-55,940 (1:200 dilution) that will be used for the stimulation. The tube containing the buffer without agonist will be used to determine the basal signal.

8. Drain the slides. Then, place them on the bench dividing them into two groups:
   (a) Stimulated slides (e.g., odd numbers).
   (b) Basal slides (e.g., even numbers).
9. Incubate the slides in assay buffer containing 3 mM GDP and 0.04 nM [^{35}S]GTPγS with (stimulated) or without (basal) 5 μM of CP-55,940 at room temperature for 2 h (*see* **Note 11**).
10. To remove unbound radioactivity, transfer the slides into racks and rinse twice in 50 mM Tris–HCl at 4 °C for 5 min, and once in deionized water (*see* **Note 12**).
11. Rapidly dry the slides under a stream of cold air, arrange into an autoradiography cassette, and expose to Biomax MR Kodak film for 48 h (*see* **Note 13**).
12. After exposure, develop the film manually or in automated procedure (*see* **Note 14**).

3.4 Image Analysis

1. Scan the autoradiography film using a scanner connected to a PC running Microsoft Windows.
2. Analyze the images with image analyzer programs such as Image-Pro Plus 5.0 (MediaCybernetics, Silver Spring, USA) or NIH Image J (*see* **Note 15**).
3. Conduct all statistical analyses using GraphPad Prism® (La Jolla, CA, USA) for Microsoft Windows, or equivalent program.

4 Notes

1. When performing the experiment, check carefully the pH of the solution.
2. When used in the protocol, this buffer produces final concentrations in the incubation reaction mixture of 50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, and 10 mU/ml adenosine deaminase.
3. One inconvenient aspect of the assay is the short physical half-life (87.4 days) of [^{35}S]GTPγS. Check the fresh lot days on the website of the supplier.
4. These solutions must be prepared and stored in the dark.
5. The gelatine solution can be used for several times; store at 4 °C and bring at room temperature before use. Slides need to be coated with gelatine to enhance adhesion of the tissue and to prevent or reduce the loss of sections during the treatment. Coating must be done carefully to avoid air bubbles.
6. You can collect coronal and/or sagittal sections. We suggest to collect sagittal sections for an overview of CB_1 receptor-stimulated

GTPγS throughout the brain. Instead, to obtain more detailed information on specific brain regions or nuclei, use coronal sections.

7. Warning: To obtain optimal signal do not use brain sections older than 6 months, unless perfectly stored at −80 °C. Never use sections older than 1 year.
8. The PAP PEN creates a hydrophobic barrier when a circle is drawn around tissue sections. This barrier stops spreading and reduces waste. The target area drawn around the sections must be the same for all slides, 400 μl for 2.5 cm × 4 cm, to allow the same concentration of reagents.
9. Pay attention to $MgCl_2$ concentration in the assay buffer, since its change can affect the outcome of the assay.
10. This solution will be used for the next incubations (Fig. 1). This assay requires a large excess of GDP to ensure that G proteins are present in the inactive state. This is needed to suppress basal binding and is reached with 1–5 mM GDP. This will allow to obtain clear section images on the autoradiographic film. When sections are too dark, try to increase GDP concentration.
11. Pay attention! Slides must not dry during this incubation. Surround slides with wet paper to maintain humidity.
12. Prepare 200 ml of 50 mM Tris–HCl per wash.
13. Autoradiography film must be handled under proper safelight condition. Make sure that the slides are completely air-dried before exposure. The emulsion coating is on a single side of the film. Film must be positioned so that the emulsion side is in contact with the slide.
14. Automated processing: Place the film into automatic processor. See the manual for your instrument. Manual processing: transfer

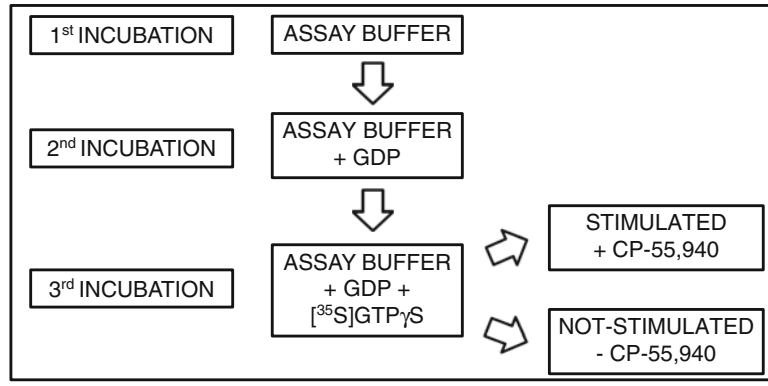

Fig. 1 Main steps of the GTPγS assay

the film in developer for 4 min with moderate agitation. Repeat this procedure with fixative and water. Dry the film.

15. Calibrate the software so that grey-level range is between 0 and 255. Trace each cerebral area with the mouse cursor control, and calculate the signal intensity (in terms of grey levels) for each traced area. For each slide also calculate the grey level of its background. Signal intensity for each region is then calculated by subtracting the value of background from each measured region. This grey level will be established within the linear range, determined by using ^{35}S standards made in the laboratory (or ^{14}C standards). For each pair of slide (stimulated/basal) calculate the percent of net stimulation as follows: (value of stimulated/value of basal) × 100.

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