Chapter 6

The Displacement Binding Assay Using Human Cannabinoid CB_2 Receptor-Transfected Cells

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Abstract

Displacement binding assays are nonfunctional assays mostly used with the aim of determining whether a certain compound (plant-derived or synthetic) is able to bind to a specific receptor with high affinity. Here, we describe the displacement binding assay that is carried out with a radioligand and CHO (Chinese Hamster Ovarian) cells stably transfected with the human cannabinoid CB_2 receptor.

Key words Binding, Cannabinoid CB_2 receptors, Agonist, Antagonist, Ligand, Radioligand, Affinity

1 Introduction

The aim of radioligand-binding experiments is to study the binding of drugs to specific receptors. Drugs that bind to a receptor and mimic the action of an endogenous ligand by inducing a biological response are defined as *agonists*. On the other hand, *competitive antagonists* are also able to bind to a receptor, but unlike agonists, they do not induce a biological response themselves, but instead, block the action of an agonist or endogenous transmitter. It is difficult to differentiate agonists from antagonists by using radioligand displacement binding assays; thus such compounds are referred to in these assays as *ligands* [1]. To perform displacement binding assays, the values of two parameters need to be known: (1) the *equilibrium dissociation constant* (K_D), which is the concentration of ligand that will occupy 50 % of the receptors, and (2) the B_{max}, which is the total density of the receptors under investigation in that preparation. Specifically, the K_D (unit M) gives an idea about the strength of the binding of a ligand to its receptor; the lower this value, the higher the affinity of the ligand for that particular receptor. The B_{max} is expressed as amount of ligand bound/mg of protein. Importantly, the determination of the maximum density of receptors in a particular tissue can be done using

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a radioligand binding assay. The values of both K_D and B_{max} can be determined using "Saturation Binding Assays," detailed descriptions of which can be found elsewhere [2, 3]. Here we describe the displacement binding assay that is carried out using Chinese Hamster Ovarian (CHO) cells stably transfected with the human cannabinoid CB_2 receptor. This assay requires the use of a radioligand, and hence can only be performed by suitably qualified researchers (registered radiation workers).

Ideal candidates for radiolabeling are compounds with: (a) high affinity for the receptor under investigation (K_D in the low nanomolar range), (b) low nonspecific binding, (c) receptor selectivity, and (d) high specific activity that will increase the sensitivity of the assay. In experiments directed at determining the affinity of certain ligands for cannabinoid CB_2 receptors, we use tritiated CP55,940 (also known as [^{3}H]CP55,940). Thus, CP55,940 is a well-established, high affinity ligand for these receptors that does however lack selectivity (it also binds potently to the cannabinoid CB_1 receptor). Because of this nonselectivity, it is recommended that binding assays with [^{3}H]CP55,940 are performed with, for example, CB_2-transfected cells, or their membranes, rather than with tissues that naturally express both CB_1 and CB_2 receptors. The method used in our laboratory to perform displacement binding assays is very simple and involves three main steps: (1) cell culturing and scraping; (2) performance of the assay; and (3) analysis of the results.

2 Materials

2.1 Cell Culturing

1. Cells: CHO cells transfected with cDNA encoding human CB_2 receptors are commercially available (e.g., ValiScreen Cannabinoid CB_2 (human) cell line, Perkin Elmer, USA).
2. Culture medium: use sterile Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, and 0.6 % G418 [3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl[oxy-2-hydroxycyclehexy]oxy-2-(1-hydroxyethyl0oxane-3,4-diol;400 mg/ml].
3. Nonenzymatic cell dissociation solution: dissolve 1 mM EDTA in distilled water, and then sterilize this solution by using an autoclave (*see* **Note 1**).
4. Use phosphate buffer saline (PBS); this is commercially available.
5. For cell culturing use plastic flasks (surface cell culture: 80 cm^2).
6. To expand the cells, use plastic flasks (surface cell culture: 175 cm^2).

2.2 Radioligand Displacement Binding Assay

This assay is carried out in nonsterile conditions; thus, all the following solutions are nonsterile.

1. Test compounds: dissolve the compounds under investigation in the appropriate solvent (e.g., dimethyl sulfoxide; DMSO) at a concentration of 10 or 100 mM, splitting the solution into 50 μl aliquots and storing each aliquot at −20 °C (*see* **Note 2**).
2. Prepare the binding/washing buffer (Tris/BSA buffer) in distilled water as follows: 50 mM Tris HCl, 50 mM Tris Base, 0.1 % w/v bovine serum albumin (BSA), pH 7.4.
3. To target cannabinoid CB_2 receptors, use [^{3}H]CP55,940 as the radioligand (e.g., provided by PerkinElmer).
4. To determine the amount of nonspecific binding, prepare a solution of CP55,940 in DMSO at a concentration of 1 mM.
5. To perform the assay, use plastic 96 deep-well plates.
6. The assay is stopped by vacuum filtration, using a 24-well sampling manifold (e.g., Brandel Cell Harvester) and Brandel GF/B filters (*see* **Note 3**).
7. To quantify the radioactivity, the filters are oven-dried (85 °C, 60 min) and then placed in 5 ml of scintillation fluid (e.g., Ultima Gold XR, PerkinElmer) and counted by liquid scintillation spectrometry (Beta counter).

3 Methods

3.1 Cell Culturing

Perform the following steps under sterile conditions.

1. Monolayers of human CB_2-transfected CHO cells are maintained at 37 °C and in 5 % CO_2 in medium flasks (surface cell culture: 80 cm^2), and are passaged twice weekly using a nonenzymatic cell dissociation solution (1 mM EDTA).
2. When cells reach 80–85 % confluence, remove medium from the flask, wash the cells with 10 ml of PBS, add 5 ml of nonenzymatic cell dissociation solution, place the flask in an incubator (at 37 °C) for 2–3 min, gently detach the cells, and add 5 ml of culture medium.
3. Expand cells by adding 2 ml of medium (containing the cells) to 25 ml of culturing medium by using four big flasks (surface cell culture: 175 cm^2). To keep the cell line, add 1 ml of the medium containing the cells to 25 ml of culturing medium in a medium flask (surface cell culture: 80 cm^2).
4. Place both medium and big flasks in an incubator (37 °C and in 5 % CO_2) until confluence is reached (*see* **Note 4**).
5. When confluence is reached (80–85 %), remove cells from the flasks by scraping and then freeze them as pellets at −20 °C until they are required.

6. On the day of the experiment, defrost cells, dilute in Tris-buffer that does not contain BSA (50 mM Tris HCl and 50 mM Tris Base), and homogenize with a 1-ml handheld homogenizer.
7. Perform a protein assay (e.g., by using a Bio-Rad Dc kit, Hercules, CA, USA) (*see* **Note 5**).

3.2 Displacement Binding Assay

1. The assay is carried out using 96 deep-well plates in a total volume of 500 μl, using Tris/BSA buffer.
2. Using Tris/BSA buffer and stock solutions of each test compound (e.g., 10 mM in DMSO), prepare decreasing concentrations of the test compound so that a dose–response curve can be drawn (*see* **Note 6**). Also prepare a *control* solution by using Tris/BSA buffer, with the same % of vehicle (e.g., DMSO) present in each diluted solution (e.g., 0.1 %).
3. Using Tris/BSA buffer and a stock solution of CP55,940 (1 mM), prepare a solution of CP55,940 (10 μM) that will be used to determine the nonspecific binding (concentration in the assay is 1 μM).
4. Prepare [^{3}H]CP55940 in Tris/BSA buffer at the appropriate concentration (*see* **Note 7**).
5. Using Tris/BSA buffer, prepare a solution of proteins (from the pellet of hCB_2-transfected cells) so that a known amount of proteins can be added (in our experiments we use 25 μg of proteins) (*see* **Note 8**).
6. Pipette 350 μl of Tris/BSA buffer, 50 μl of [H^3]CP55940 at an appropriate concentration (e.g., 0.7 nM), 50 μl of varying concentrations of the test drug, and 50 μl of cell membranes at an appropriate dose (e.g., 25 μg) into the wells of a 96 deep-well plate, progressing along each row from left to right.
7. Incubate the 96 well plates at 37 °C in a darkened water bath for 60 min.
8. Terminate the assay by adding ice-cold Tris/BSA buffer and then performing rapid vacuum filtration with a 24-well sampling manifold (e.g., Brandel Cell Harvester), and Brandel GF/B filter papers that have been soaked in Tris/BSA buffer at 4 °C for 24 h.
9. Wash each well 3/4 times with Tris/BSA buffer.
10. Dry the filter papers in an oven for 60 min at 85 °C.
11. Place each of these filter papers in a different scintillation vial and add 4 ml of scintillation fluid.
12. After soaking each filter paper in the scintillation fluid for 60 min, measure radioactivity with a beta counter. Values will be in cpm (counts per minute).

3.3 Analysis of the Results

1. The percentage (%) of "specific binding" is calculated as follows:

$$\frac{\text{mean cpm for control} - \text{mean cpm for CP55,940}\,(1\ \text{M})}{\text{mean cpm for control}} \times 100$$

2. The percentage of any displacement produced by each concentration of the test compound is calculated as follows:

$$\frac{\text{mean cpm for control} - \text{mean cpm for ligand at a particular concentration}}{\text{mean cpm for control} - \text{mean cpm for CP55,940}\,(1\ \text{M})} \times 100$$

4 Notes

1. The cell dissociation solution is also commercially available.
2. Avoid multiple thawing of the same aliquot, since this may result in the degradation of chemically unstable compounds. We recommend that the same aliquot is not used more than twice.
3. Before use, filters are soaked in Tris/BSA buffer at 4 °C for at least 24 h.
4. CHO cells grow up quickly. Usually, confluence is reached after 3 days.
5. The protein assay can be carried out as follows:
   (a) Prepare standards and unknown(s) (*X*) as shown in the table (Table 1) below (this scheme is suitable for a Biorad Kit).
   (b) Add the KIT reagents (as described by the manufacturer).
   (c) After 15 min read absorbance at 750 nm.
   (d) Construct the BSA standard curve by using the absorbance values of each standard solution (see table above), in order to perform linear regression analysis (e.g., using GraphPad Prism software).
   (e) Determine the concentration (mg/ml) of proteins in your sample (*X*), by using the above BSA standard curve to interpolate the absorbance value of your sample, then multiply the value obtained by 2 (this is the cell dilution factor; e.g., as described above, we added 50 μl of Tris-buffer to 50 μl of cell solution, thus we have diluted the cells by a factor of two).
   (f) Make a solution containing cells at a known concentration for use in the assay.

Table 1
BSA standard curve for the quantification of the content of unknown proteins

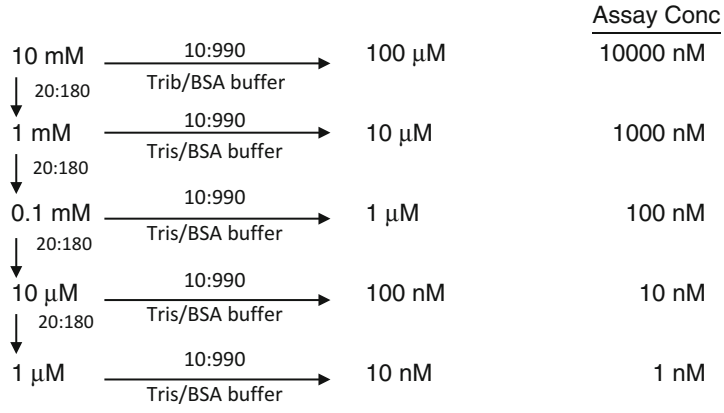
	A	B	C	D	E	F
Conc (mg/ml)	1.5	0.75	0.45	0.3	0.15	*X*
Standard BSA (μl)	100	50	30	20	10	50
Tris-Buffer (no BSA) (μl)	–	50	70	80	90	50

Please note that: (1) A–E are the concentrations of BSA used to build the BSA standard curve, which in turn is used to determine the concentration of proteins in your sample, and (2) by adding 50 μl of Tris-Buffer to 50 μl of cell solution, your sample is diluted twice and this should be taken into account in the calculation of the cell sample concentration

6. To construct a log concentration-response curve for the compound under investigation, prepare a stock solution by dissolving the test compound in DMSO (e.g., 10 mM), and then making serial dilutions to obtain solutions that have one of the following concentrations (*X*): 10, 1 μM, 100, 10, and 1 nM, ensuring that the concentration of vehicle in each solution remains constant (e.g., 0.1 %). Please note that: (1) each of these concentrations should be 10*X*, since 50 μl of each solution is added in the assay in a final volume of 500 μl (thus the solution will be diluted 10 times), and (2) in a sigmoidal log concentration-response curve, these concentrations are expressed as log M (Fig. 1).

   Example of Drug Dilutions (from a 10 mM stock solution in DMSO)

   Please note that the dilutions indicated on the left side of the scheme below should be made by using your stock solution solvent (in this case, DMSO).



7. The concentration of [^{3}H]CP55940 we use in our experiments is 0.7 nM. The binding parameters (B_{max} and K_D) for [^{3}H]CP55940 are determined by fitting data from saturation-

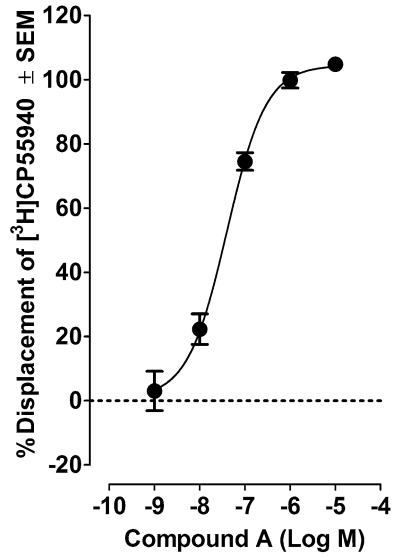


Fig. 1 Displacement of [^{3}H]CP55940 by compound A from specific binding sites on hCB_2 CHO cell membranes. Each symbol represents the mean percentage displacement ± SEM

binding experiments to a one-site saturation plot by using GraphPad Prism. For commercially available cells stably transfected with human CB_2, like the ones we use in our laboratory, these parameters are provided by the manufacturer.

8. Before any displacement binding assay is carried out, the protein content should be optimized by testing increasing concentrations of proteins versus a constant concentration of [^{3}H] CP55,940.

References

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