# G Protein-Coupled Receptors

## Essential Methods

Editors

**David R. Poyner**

*School of Life and Health Sciences, University of Aston, Birmingham, UK*

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# Contents

# Preface

This book describes a number of techniques relating to research on G protein-coupled receptors (GPCRs), written by a number of leading international authorities. In line with the rest of the essential methods series, each chapter contains an overview of the method and this is followed by a series of detailed protocols, providing a bench-side guide.

The literature on GPCRs is vast and the pace of investigation shows no sign of slackening. Given that these receptors are not only the largest protein family in the human genome but are also the single biggest target for therapeutic agents, this level of interest is not surprising. For any editor, this poses problems; the range of techniques that can be applied to GPCRs is vast and continues to grow, so it is impossible to cover them all in a single volume. In the case of this book, the techniques that are covered have been selected simply because we think that they will prove to be useful tools in future research and will contribute to increasing our understanding of GPCRs. In addition, they are of interest to the editors. Doubtless, readers wishing to find descriptions of additional methods will be able to find these elsewhere in the GPCR literature. Some of the techniques covered in this volume are very well known, such as mutagenesis or measurement of second messengers. We have included these because they are so fundamental to GPCR research and so might be of interest to the newcomer to the field. Some of the other techniques presented might not be so widely employed, but they have proved their worth in numerous laboratories.

Chapters 1 and 2, by Leach *et al*. and Gregory *et al*. respectively, review radioligand binding techniques (including detection of allosteric modulators) and measurements of a range of second messengers activated by GPCRs. Measurement of GTP$\gamma$S binding as an index of receptor activation is dealt with by Kara and Strange in Chapter 3. Quantitative GPCR imaging is considered in the context of receptor trafficking by James *et al*. in Chapter 4. In Chapter 5, Darby *et al*. describe methods for overexpressing GPCRs, concentrating on *Pichia pastoris* as a host. In Chapter 6, Jaeger *et al*. consider how bioluminescence resonance energy transfer can be used to look at GPCR complexes, whereas Krasel and Hoffmann describe in Chapter 7 how fluorescence resonance energy transfer can be used to measure receptor conformation. Chapters 8 and 12, by Li *et al*. and Liapakis and Javitich respectively, review the utility of engineered cysteines for artificial intramolecular disulfide bonds and cysteine scanning accessibility mutagenesis respectively. The new technique of fluorescence correlation spectroscopy

is described by Briddon *et al*. in Chapter 9. Mundell *et al*. (Chapter 11) and Kong *et al*. (Chapter 10) consider receptor regulation; how this can be quantified and how GPCR phosphorylation can be measured. Simms reviews techniques for receptor modelling in Chapter 13 and the Appendix (by Conner *et al*.) considers methods for mutagenesis.

We hope this volume will be useful to investigators in GPCR research.

**David R. Poyner**
**Mark Wheatley**

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# 1

# Measurement of Ligand–G Protein-coupled Receptor Interactions

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## 1.1 Introduction

### 1.1.1 Ligand–receptor interactions and the law of mass action

Radioligand binding assays take advantage of the ability to detect the decay of radioactive material, which can be incorporated into a ligand of choice. The interaction of such a radioligand with a receptor preparation can subsequently be determined by capturing and measuring the amount of radioactivity present. Radioligand binding assays can be used to estimate molecular parameters, such as the density of receptors present in a tissue or cellular preparation or the affinity of a ligand for binding to a receptor.

The simplest scheme that describes the binding of a ligand to its receptor is based on the law of mass action:

$$A + R \underset{K_{off}}{\overset{K_{on}}{\rightleftharpoons}} AR$$

where the ligand A binds to the receptor R to form the ligand–receptor complex AR. The rate at which the ligand binds to the receptor, expressed as the number of binding events per unit of time, is dependent on the ligand concentration, the number of

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unoccupied receptors and the association rate constant $K_{on}$. In contrast to an enzymatic reaction, there is no degradation of the product AR; and if this reaction is reversible, then the ligand–receptor complexes can dissociate into free receptor and ligand, which is dependent on the concentration of ligand–receptor complexes and the dissociation rate constant $K_{off}$. Although the association and dissociation rates may differ, over time an equilibrium state will arise whereby the rate at which new ligand–receptor complexes are formed will equal the rate at which ligand–receptor complexes break down. At equilibrium, the ratio of the dissociation and association rate constants provides a useful measure of the overall strength with which a ligand interacts with a receptor, the equilibrium dissociation constant $K_a$ (although sometimes referred to as $K_d$) and expressed in moles per litre. $K_a$ is also the concentration of ligand that binds half the receptors present. Therefore, at equilibrium, the concentration of ligand–receptor complexes is governed by the total receptor density $[R_T]$, the ligand concentration [A] and the equilibrium dissociation constant of the ligand:

$$[AR] = \frac{[R_T] \times [A]}{[A] + K_a} \tag{1.1}$$

where $[R_T] = [R] + [AR]$ and $K_a = K_{off}/K_{on}$.

Equation 1.1 is often referred to as the Hill–Langmuir binding isotherm and describes equilibrium binding of a ligand to a receptor under the law of mass action, such that a hyperbolic curve will result when binding is plotted against the molar ligand concentration. This was first used by A.V. Hill to describe the binding of oxygen to haemoglobin [1, 2]. If ligand concentrations are expressed in logarithmic space, then a sigmoidal concentration–occupancy curve will be apparent. If binding of a ligand to a receptor at equilibrium follows a simple mechanism, where the binding of one ligand molecule is unaffected by concomitant binding events and where the ligand binds to only a single class of receptor sites, then the concentration–occupancy relationship plotted on a logarithmic scale will follow a sigmoidal curve that approximately spans from 10 to 90% occupancy over a 100-fold, or two log-unit, concentration range. The steepness of the slope of such a curve in linear space, the Hill coefficient, will equal unity.

## 1.1.2 Competitive interactions at G protein-coupled receptors

### *1.1.2.1 Antagonist binding*

Although ligand–G protein-coupled receptor (GPCR) interactions can be quantified by observing the binding of a radiolabelled ligand to a receptor, it is sometimes more practical to measure the ability of a fixed concentration of radioligand to bind to the receptor in the presence of increasing concentrations of an unlabelled ligand, to indirectly determine the interaction of the unlabelled ligand with the receptor. If we consider the binding of ligand A in the presence of a competitor B at equilibrium:

$$A + BR \underset{K_{off\,B}}{\overset{K_{on\,B}}{\rightleftharpoons}} A + R + B \underset{K_{off\,A}}{\overset{K_{on\,A}}{\rightleftharpoons}} AR + B$$

then we can define the concentration of receptors bound to the radiolabelled ligand A in the presence of an unlabelled competitive ligand B, which was first derived by Gaddum [3, 4]. A form of this relationship can be described by

$$[\mathrm{AR}] = \frac{[\mathrm{R_T}] \times [\mathrm{A}]}{[\mathrm{A}] + K_\mathrm{a}\left(1 + \dfrac{[\mathrm{B}]}{K_\mathrm{b}}\right)} \tag{1.2}$$

Competitive binding data are commonly expressed as the fractional inhibition of radioligand binding in the presence of the competitor. However, the binding of some ligands does not follow the simple law of mass action and receptor occupancy is not always directly proportional to ligand concentration (see Section 1.1.2.2). Under these circumstances, the Hill slope may vary from unity and must, therefore, be empirically incorporated into any ligand binding equation to derive the steepness of the slope describing the concentration–occupancy relationship, as shown in Equation 1.3:

$$Y = \frac{\mathrm{Top} - \mathrm{Bottom}}{1 + \dfrac{[\mathrm{B}]^n}{\mathrm{IC}_{50}}} + \mathrm{Bottom} \tag{1.3}$$

where $Y$ is radioligand binding, Top is the top asymptote of the curve equal to total binding of the radioligand in the absence of competitor B, Bottom is the bottom asymptote of the curve equal to nonspecific binding, $n$ is the Hill coefficient and $\mathrm{IC}_{50}$ is the concentration of B that inhibits 50% of radioligand binding. If the Hill coefficient equals unity, then the equilibrium dissociation constant of the unlabelled ligand $K_\mathrm{b}$ can be determined using the Cheng–Prusoff equation [5]:

$$K_\mathrm{b} = \frac{\mathrm{IC}_{50}}{1 + \dfrac{[\mathrm{A}]}{K_\mathrm{a}}} \tag{1.4}$$

#### *1.1.2.2 Agonist binding*

Although binding of an antagonist to a receptor will often display a concentration–occupancy relationship that has a Hill coefficient of unity, agonist binding to GPCRs is usually more complex. This has been well characterized in competition assays between agonists and radiolabelled antagonists, which often yield shallow curves, with Hill coefficients less than unity. These shallow curves reflect different receptor states, for which the agonist has different affinities and the (often) biphasic nature of the curves gives rise to a competition curve that spans greater than a twofold concentration range of competitor (see Figure 1.1).

The addition of guanine nucleotides such as guanosine diphosphate, guanosine triphosphate (GTP), guanylylimidodiphosphate (GppNHp) and guanosine 5′-*O*-(3-thiotriphosphate) (GTP$\gamma$S) often alters the proportion and affinity of the two binding sites, demonstrating that the dispersion of agonist affinity states reflects the formation of a ternary complex consisting of an agonist, a GPCR and a guanine

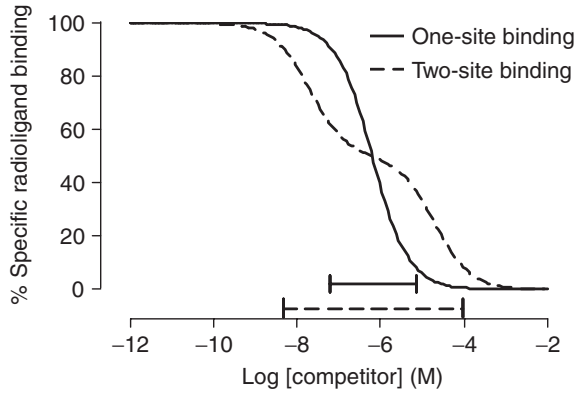


**Figure 1.1** Theoretical competition binding curve simulated using a one-site binding fit that follows a Hill slope of unity (solid line) in comparison with a two-site binding fit that follows a shallow slope (dashed line).

nucleotide binding protein (G protein). A ternary complex model (TCM) has been proposed to explain the shallow curves observed with agonists versus radiolabelled antagonists in competition binding assays, but requires a number of assumptions that are not often met [6]. For instance, it must be assumed that the G protein is limiting so that not all of the receptors can form a complex with the G protein, enabling both G protein-coupled (high-affinity) and -uncoupled (low-affinity) receptor species to be observed. This is rarely observed in cellular systems used to study many ligand–GPCR interactions, where G protein levels often exceed those of receptor expression levels.

More sophisticated extensions of the TCM have been developed to account for the ability of agonists to bind with higher affinity to receptors that have been mutated to exert constitutive activity than to their wild-type counterparts, even in the absence of G proteins [7]. However, in general there is little advantage to using the extended TCM for routine data analysis, as the simpler TCM can adequately approximate the binding of agonists to a receptor.

### 1.1.3 Allosteric ligands

For a number of GPCRs, there are ligands that can bind to the receptor at a site that is topographically distinct from the endogenous, or orthosteric, ligand binding site [8]. These binding sites and the ligands that bind to them are referred to as ‘allosteric’. Since allosteric ligands do not directly compete for binding with the orthosteric ligand, they have the ability to form a ternary complex in which both the orthosteric and the allosteric ligand occupy the receptor (see Figure 1.2).

Binding of an allosteric ligand to a receptor may alter the receptor conformation such that binding of the orthosteric ligand is altered, and vice versa. These changes in binding are termed ‘cooperative effects’ [9].

In terms of binding, the allosteric TCM predicts that an allosteric modulator may inhibit ($0 < \alpha < 1$), enhance ($\alpha > 1$) or have no effect ($\alpha = 1$) on the binding of an

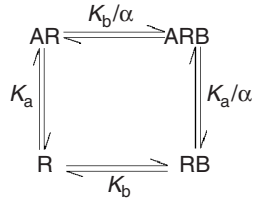


**Figure 1.2** A ternary complex model describing the binding of an orthosteric ligand A and an allosteric modulator B to a receptor R. $K_a$ and $K_b$ are the equilibrium dissociation constants of R for A and B respectively, $\alpha$ is the binding cooperativity between A and B (the selectivity of A for R and RB or of B for R and AR) and, therefore, determines the effect of B on the binding of A and vice versa.

orthosteric ligand. The formation of a ternary complex between the receptor, the orthosteric ligand and the allosteric modulator can be described by

$$[\mathrm{AR}] + [\mathrm{ARB}] = \frac{[\mathrm{R_T}] \times [\mathrm{A}]}{[\mathrm{A}] + K_a\left[\left(1 + \frac{[\mathrm{B}]}{K_b}\right) \Big/ \left(1 + \frac{\alpha[\mathrm{B}]}{K_b}\right)\right]} \tag{1.5}$$

Several recent reviews provide detailed information regarding our understanding of such compounds and the methods that can be used to detect and analyse allosteric interactions [8, 10, 11].

## 1.2 Methods and approaches

### 1.2.1 General considerations for radioligand binding assays

#### *1.2.1.1 Buffers*

Acidic, basic or neutral buffers, consisting of pH buffering agents such as trishydroxymethylaminomethane hydrochloride (tris acid), trishydroxymethylaminomethane (tris base) and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), are all commonly used for radioligand binding assays, usually at concentrations of 10–50 mM. HEPES-based buffers are popular as HEPES is relatively heat stable. For some receptors, ligand binding and receptor activity are highly dependent upon pH, particularly as both the receptor and the ligand may be protonated (acidified) or deprotonated depending on the pH. Many researchers chose to perform radioligand binding assays at pH 7.4, which resembles a physiological environment.

The composition of the buffer is particularly important, as certain trace metals can directly interact with GPCRs and alter their behaviour. For instance, 100 mM NaCl is commonly used to maintain a high ionic strength, yet sodium can destabilize receptor–G protein formation by interacting with a highly conserved aspartate residue in transmembrane domain 2 of Family A GPCRs [12], which can have implications for the binding of agonists and inverse agonists. Sodium may also have less specific effects that are due to the ionic strength of the buffer, and comparing the effects of sodium

and potassium may be useful to determine the potential impact of this phenomenon. Alternatively, 100 mM *N*-methyl-D-glucamine may be substituted for sodium to alter ionic strength whilst having no effects on receptor–G protein coupling. Additional ions may also have direct effects on ligand binding or receptor activity. For instance, magnesium is required for efficient coupling between the receptor and G protein and may, therefore, enhance the proportion of high-affinity agonist binding sites. Magnesium is usually present in the buffer at concentrations between 1 and 10 mM, a concentration that can also be used in functional membrane-based assays such as [$^{35}$S]GTP$\gamma$S binding assays. Calcium can directly bind to members of the Family C GPCRs to alter the binding of ligands [13] and can also activate certain proteases that may enhance degradation of the receptor. Ethyleneglycoltetraacetate (EGTA) may be added to the assay buffer to chelate calcium ions and act as a protease inhibitor, whilst ethylenediaminetetraacetate (EDTA) is useful for chelating additional trace metals, such as magnesium, to prevent ligand oxidation. For these reasons, EDTA is a useful component in the buffer used to prepare membranes for radioligand binding (see Section 1.2.2.1). Additional protease inhibitors may also be added, such as serine-, cysteine- and metallo-protease inhibitors.

Finally, the buffer must also be optimal for the ligands to be used in the radioligand binding assay. Some ligands, particularly 5-hydroxytryptamine and catecholamines such as dopamine, epinephrine and norepinephrine, are particularly susceptible to oxidation, so an antioxidant such as ascorbic acid can be added to the buffer to prevent this. Other ligands, such as proteins and peptides, may stick to plastic and glass, and a protein such as bovine serum albumin (0.001–0.1%) may be required to coat these surfaces to reduce adsorption of the ligand.

### *1.2.1.2 Temperature and incubation time*

Radioligand binding assays are usually performed at 20–37 °C. Although 37 °C is physiologically relevant, receptors studied in membrane systems may become unstable at high temperatures. If lower temperatures are used, then care must be taken to ensure that equilibrium binding is reached. Therefore, the temperature at which the experiment is performed depends upon the stability of the receptor and ligands and the binding kinetics of the ligand. The law of thermodynamics predicts that equilibrium will be reached faster at higher temperatures, with an approximate doubling in the reaction rate with a 10 °C increase in temperature.

With regard to incubation time, this may vary between receptors. The binding of a fixed concentration of radioligand should initially be measured at different time points to determine when equilibrium is attained (see Section 1.2.2.5). The rate at which the ligand–receptor interaction approaches equilibrium is often termed the $K_{\text{observed}}$ or $K_{\text{obs}}$, measured in units of inverse time. $K_{\text{obs}}$ is dependent on the association and dissociation rate constants of the ligand and the ligand concentration, with lower ligand concentrations taking longer to reach equilibrium. Thus, binding of a radioligand to a receptor starting from time point 0 will follow

$$Y = [\text{AR}]_{\text{Eq}} \times (1 - \text{e}^{-K_{\text{obs}}t}) \tag{1.6}$$

where $K_{obs} = K_{on}[A] + K_{off}$ and $[AR]_{Eq}$ is binding once equilibrium has been reached (expressed in units of the $Y$ axis, such as disintegrations per minute (dpm)).

Low ligand concentrations should, therefore, be used when testing equilibration time. It is recommended that five times the dissociation half-life of any ligand should be allowed in order to reach approximately 97% equilibrium binding with the receptor, which is considered to be sufficient.

### *1.2.1.3 Ligand depletion*

Under ideal experimental conditions only a small fraction of the total ligand added will bind to the receptor or to nonspecific sites. Thus, the free-ligand concentration throughout the assay is generally close to the total concentration of ligand added to the assay. However, if a large proportion of the ligand added is bound, either specifically or nonspecifically, then the concentration of free ligand in solution will deviate significantly from the concentration added to the assay. Generally, if less than 10% of the ligand is bound at each given ligand concentration, then ligand depletion is minimal and, therefore, is not a concern. Initial experiments can be carried out to optimize the protein content of the assay and ensure that excess radioligand does not bind to the protein added. This can be achieved by measuring the binding of a fixed radioligand concentration in the presence of different concentrations of the receptor preparation and calculating the percentage of radioligand bound at each receptor concentration. If ligand depletion does appear to be a problem, then the assay format may be altered to overcome this. The assay volume may be increased, but a greater amount of ligand will also be required to obtain the same concentration whilst receptor numbers will remain constant. Alternatively, the free radioligand can be measured in each tube if a centrifugation assay is employed (see Section 1.2.1.4). Otherwise, analysis techniques that account for the differences between the added and free ligand concentration can be used (see Section 1.2.2.2).

### *1.2.1.4 Separation of bound from free radioligand*

In order to measure the amount of radioligand bound to the receptor preparation, bound radioligand must usually be separated from free radioligand. The most common separation method is vacuum filtration, whereby the samples are rapidly filtered, generally onto glass-fibre filter paper, and washed to remove radioligand that is weakly bound to the filter or to the receptor preparation. Be aware that high concentrations of membrane can clog the filter pores, leading to slower filtration and washing rates. The wash buffer should be ice-cold and washing should be rapid so as to minimize dissociation of the radioligand from the receptors. Filtration of samples can, however, lead to loss of receptors, as some can pass through the glass-fibre filters. High-speed centrifugation assays may be used to minimize loss of receptor and ensure that a greater proportion of the ligand-bound receptor is collected. However, these assays may require more protein in order to pellet the membrane efficiently. The supernatant can then be removed and the pellet rapidly washed. Centrifugation assays are also useful when ligand depletion is a problem, as the free radioligand concentration can be determined following termination of the assay.

## 1.2.2 General assay protocols

### *1.2.2.1 Membrane preparations*

Radioligand binding may be studied in a cell line or tissue that endogenously expresses the receptor of interest, or a recombinant cell system in which receptor expression is induced. These assays may be performed on whole cells, on tissues or on soluble and purified receptors. However, a common approach is to prepare membrane preparations from the cell lines or tissues expressing the receptor (Protocol 1.1). Although the generation of membranes can be more expensive than using live cells, the use of membrane preparations is particularly convenient because the membranes can be frozen and stored for several months and defrosted when required. It is recommended to perform each repeat of a radioligand binding assay on a new membrane preparation made from a new tissue preparation or cell passage number.

### PROTOCOL 1.1 Preparation of Membranes from Adherent Mammalian Cells

#### Equipment and Reagents

- Lifting buffer; for example, 2 mM EDTA in a phosphate-buffered saline (PBS) solution (150 mM NaCl, 16 mM $Na_2HPO_4$, 4 mM $NaH_2PO_4$) for Chinese hamster ovary (CHO) cells, or PBS alone for less adherent cell lines, such as HEK 293 cells
- HEPES-based buffer; for example, 20 mM HEPES, 10 mM EDTA, pH 7.4
- Low-EDTA HEPES-based buffer; for example, 20 mM HEPES, 0.1–1 mM EDTA, pH 7.4
- Low- and high-speed chilled centrifuges
- Homogenizer.

#### Method

1 Grow cells to 80–90% confluence in T175 flasks (175 $cm^2$). Generally, one T175 flask will provide sufficient membrane for approximately 50 assay tubes using 15 µg of protein per tube. Remove media and add 10 ml warm lifting buffer[a] to each flask. For cell lines that are particularly adherent, an initial wash with calcium-free PBS may be required to remove any remaining media. For cell lines that require lifting buffer containing EDTA, incubate for 2–5 min at 37 °C. Gently tap the flasks to detach cells and collect the cell suspension in appropriate tubes. Wash each flask with 10 ml PBS and collect the wash.

2 Centrifuge the cell suspension at approximately 200 g (approximately 1200 rpm in most bench-top centrifuges) for 10 min and resuspend the cell pellet in 2–3 ml HEPES- or tris-based buffer[b] per T175 flask, or an appropriate volume to ensure efficient homogenization (step 3).

3 All subsequent steps should be performed at 4 °C to reduce activation of proteases. Homogenize the cell solution using a homogenizer. Perform three 5–10 s bursts at top speed (~20 000 rpm) with cooling on ice in between each burst.

4 Centrifuge the resulting cell lysate at 600 g (approximately 1700 rpm in most bench-top centrifuges) for 10 min to separate the nuclear fraction and additional cell debris. If a large pellet is obtained, resuspend the pellet and repeat steps 3 and 4, combining the supernatant obtained following each centrifugation step.

5 Transfer the remaining suspension to new tubes and centrifuge at approximately 40 000 g or higher for 1 h. Resuspend the resulting protein pellet in a HEPES- or tris-based buffer,[c] which can be the buffer that will be used for subsequent radioligand binding assays, or a predominantly HEPES or tris-based buffer with a low concentration (0.1–1 mM) of a chelating agent such as EDTA or EGTA. Resuspend in approximately 0.5–2 ml buffer per T175 flask, depending on the expression level of the receptor and subsequently the amount of protein required for each assay.

6 Homogenize the membrane suspension briefly and dispense into aliquots of a suitable volume. Membranes can be stored at −80 °C, generally for up to 12 months, and the protein content of the preparation can be determined using an appropriate method such as that of Lowry [14] or Bradford [15].

### *Notes*

[a]It is not recommended to use trypsin for harvesting cells, as receptors may be hydrolysed.

[b]The HEPES- or tris-based buffer should contain 1–10 mM EDTA, EGTA or both to reduce proteolysis of the receptor of interest following homogenization steps.

[c]Ten confluent T175 flasks should generate approximately 10 ml of protein at a concentration of 1–2 mg/ml.

### *1.2.2.2 Saturation binding assays*

Saturation binding assays are used to determine the binding of different radioligand concentrations to a receptor at equilibrium directly. These assays can be used to derive direct measurements of the total receptor number or density present in the system under investigation and to determine the affinity of the radioligand for the receptor. However, the radioligand will not only bind specifically to the receptor of interest, but will also bind to additional sites within a membrane or cell preparation or to the tubes used to perform the radioligand binding assay. Therefore, nonspecific binding must be determined in parallel using a high concentration of a competing ligand to displace each radioligand concentration from the receptor. Where possible, the competitive ligand used to define nonspecific binding should not be an unlabelled form of the radioligand, as both compounds will compete for the same nonspecific binding sites. Ideally, at least 100–1000 times the $K_a$ of the competitive ligand used to define nonspecific binding should be used to ensure full receptor occupancy.

Protocols 1.2–1.6 for radioligand binding assays describe assays performed in 0.5–1 ml volumes, but are applicable to smaller volume assays that can be scaled down.

Traditionally, specific binding is determined by subtracting nonspecific binding from total binding and the data are analysed using Equation 1.7 (see Figure 1.3). However,

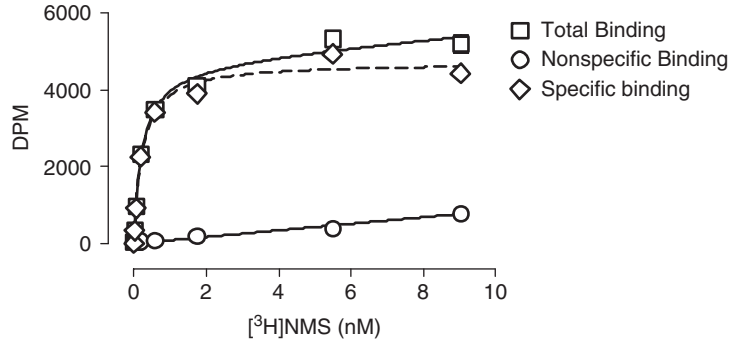


**Figure 1.3** Saturation radioligand binding. Binding of the radioligand, [$^3$H]*N*-methylscopolamine ([$^3$H]NMS), to the $M_4$ muscarinic acetylcholine (ACh) receptor stably expressed in FlpIn™ CHO cells, where nonspecific binding was determined in the presence of 10 μM atropine. Membranes were incubated with [$^3$H]NMS for 1 h at 37 °C before the assay was terminated as described in the methods section.

with the development of more sophisticated computer software, it is now possible to fit the data globally to a model of receptor and nonspecific site occupancy, shown in Equation 1.8. If data are fitted globally, then this means that a family of curves is fitted to a particular model, rather than just a single curve. Parameters common to both datasets can be shared between them, enabling their determination from the relationship between all curves (a comprehensive explanation of global fitting is provided in [16]).

The radioligand–receptor complex concentration [AR] that forms at equilibrium at each radioligand concentration is reflected by the specific binding of the radioligand, expressed on the *y*-axis. We usually call the total number of receptors that are defined by our radioligand as the $B_{max}$, so specific binding of each radioligand concentration in a saturation binding assay is defined by the Hill–Langmuir occupancy equation using the terms shown in Equation 1.7. Note the relationship to Equation 1.1.

$$Y = \frac{B_{max} \times [A]}{[A] + K_a} \tag{1.7}$$

Nonspecific binding is generally linearly proportional to the radioligand concentration and, therefore, is described by the equation for a straight line. As the total binding TB measured in the experiments represents both specific and nonspecific binding, radioligand binding, expressed on the *y*-axis, to one class of binding sites is defined by

$$\mathrm{TB} = \frac{B_{max} \times [A]}{[A] + K_a} + \mathrm{NS} \times [A] \tag{1.8}$$

where NS is the nonspecific binding.

In the presence of ligand depletion, binding is defined by

$$\mathrm{TB} = \frac{B_{max} \times ([A]_T - \mathrm{TB})}{([A]_T - \mathrm{TB}) + K_a} + ([A]_T - [\mathrm{TB}]) \times \mathrm{NS} \tag{1.9}$$

where $[A]_T$ is the total radioligand concentration added. As TB appears on both sides of Equation 1.9 (implicit equation), it cannot be entered into most nonlinear regression programs. The equation can, however, be rearranged into a quadratic equation:

$$TB = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \tag{1.10}$$

where

$a = -1 - NS$
$b = [A]_T[2(NS + 1)] + K_a(NS + 1) + B_{max}$

and

$c = -[A]_T[NS(K_a + [A]_T)] + B_{max}$

If total and nonspecific binding data are fitted globally, then nonspecific binding should be shared between datasets so that the fraction of total binding that is nonspecific at each radioligand concentration can be determined. This type of analysis will derive the $K_a$ and the maximum level of binding of the radioligand without the need to subtract nonspecific from total binding. However, the raw data values that are derived from a saturation radioligand binding assay are usually more useful if converted to the amount of radioligand bound to our receptor preparation. We usually define the total number of binding sites in reference to the amount of protein or cells. For instance, 15 µg protein were added into each tube of the saturation assay shown in Figure 1.3; therefore, 1.7 pmol of receptor is expressed per milligram of protein.

## PROTOCOL 1.2 Saturation Binding Assays

### Equipment and Reagents

- Membrane preparation (Protocol 1.1)
- Binding buffer[a]
- 5 ml polypropylene[b] assay tubes (Techno-plas)
- Radioligand
- Competitive antagonist
- Wash buffer[c]
- Water bath
- Vacuum harvester (Brandel)
- Glass-fibre filter paper[d] (Whatman)
- Liquid scintillation cocktail[e] (PerkinElmer)
- Liquid scintillation counter (e.g. Packard Tri-Carb LS counter).

### Method

1 Dilute the receptor preparation, radioligand and competitive antagonist in assay buffer to 10× the final concentrations required in the assay, so that each can be diluted by this factor when added to the final assay mix.

2 In an appropriate volume of binding buffer (usually between 0.5 and 1 ml), prepare one set of assay tubes containing increasing concentrations of radioligand and one identical set of tubes that also contain a saturating concentration of a competitive ligand to define nonspecific binding (see Table 1.1).

**Table 1.1** Tube set-up used to determine saturation binding of [$^3$H]NMS to the $M_4$ muscarinic ACh receptor using a 500 µl final assay volume, where nonspecific binding is determined in the presence of 10 µM atropine.

| Final [[$^3$H]NMS] (M) | [$^3$H]NMS (µl) | 100 µM atropine (10 µM final concentration) (µl) | Buffer (µl) | Membrane (µl) |
|---|---|---|---|---|
| *Total binding* | | | | |
| 0 | 0 | 0 | 450 | 50 |
| $1 \times 10^{-11}$ | 50 (of $1 \times 10^{-10}$ M) | 0 | 400 | 50 |
| $3.16 \times 10^{-11}$ | 160 (of $1 \times 10^{-10}$ M) | 0 | 290 | 50 |
| $1 \times 10^{-10}$ | 50 (of $1 \times 10^{-9}$ M) | 0 | 400 | 50 |
| $3.16 \times 10^{-10}$ | 160 (of $1 \times 10^{-9}$ M) | 0 | 290 | 50 |
| $1 \times 10^{-9}$ | 50 (of $1 \times 10^{-8}$ M) | 0 | 400 | 50 |
| $3.16 \times 10^{-9}$ | 160 (of $1 \times 10^{-8}$ M) | 0 | 290 | 50 |
| $1 \times 10^{-8}$ | 50 (of $1 \times 10^{-7}$ M) | 0 | 400 | 50 |
| *Nonspecific binding* | | | | |
| 0 | 0 | 50 | 400 | 50 |
| $1 \times 10^{-11}$ | 50 (of $1 \times 10^{-10}$ M) | 50 | 350 | 50 |
| $3.16 \times 10^{-11}$ | 160 (of $1 \times 10^{-10}$ M) | 50 | 240 | 50 |
| $1 \times 10^{-10}$ | 50 (of $1 \times 10^{-9}$ M) | 50 | 350 | 50 |
| $3.16 \times 10^{-10}$ | 160 (of $1 \times 10^{-9}$ M) | 50 | 240 | 50 |
| $1 \times 10^{-9}$ | 50 (of $1 \times 10^{-8}$ M) | 50 | 350 | 50 |
| $3.16 \times 10^{-9}$ | 160 (of $1 \times 10^{-8}$ M) | 50 | 240 | 50 |
| $1 \times 10^{-8}$ | 50 (of $1 \times 10^{-7}$ M) | 50 | 350 | 50 |

3 Start the binding reaction by addition of membrane protein,[f] bringing the assay to the desired volume and incubate the reaction for sufficient time so as to reach equilibrium binding (at least five times the dissociation half-life of the ligand).

4 At the appropriate time, terminate the reaction by rapid vacuum filtration through glass-fibre filter paper followed by three to four 4 ml washes with ice-cold wash buffer to separate bound from free radioligand.

5 Determine radioactivity by liquid scintillation counting.

### *Notes*

[a]For any radioligand binding assay, the buffer may be a simple buffer containing no or low concentrations of ions with chelating agents such as EDTA and EGTA (e.g. 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH 7.4) or the buffer may resemble a more physiological environment or a buffer used for functional assays such as [$^{35}$S]GTP$\gamma$S binding assays (e.g. 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). Care should be taken when choosing the appropriate acid or base to adjust the pH of the buffer. Sodium hydroxide (NaOH), for instance, will alter the concentration of sodium ions in the solution, which may have effects on the binding of ligands. 1–2 M KOH is more appropriate for adjusting the pH of radioligand binding buffers.

[b]Proteins and peptides are less likely to stick to polypropylene than to polystyrene-based plastics.

[c]PBS or other simple saline solution, such as 0.9% NaCl.

[d]GF/B-grade glass-fibre filter paper has larger pores and, therefore, is generally better suited to cell-based binding assays, whilst GF/C-grade glass-fibre filter paper is better suited for membrane-based assays. However, higher nonspecific binding may be apparent when using GF/C-grade glass-fibre filter paper.

[e]Certain scintillation cocktails may be better suited to specific applications. For instance, whilst Perkin Elmer's Ultima Gold™ is suitable for both aqueous and nonaqueous samples, Irga Safe Plus™ is more suited to aqueous samples.

[f]The appropriate protein concentration should be optimized prior to the performance of any ligand binding assay to ensure that ligand depletion does not occur.

### *1.2.2.3 Heterologous and homologous competition binding experiments*

The use of high radioligand concentrations in saturation binding experiments can be expensive, whilst many ligands are unavailable in radioactive form, meaning that their affinity for a receptor cannot always be directly determined. Competition binding assays, however, are an alternative means to study interactions between the receptor and an unlabelled ligand under equilibrium conditions, by measuring the binding pattern of a single radioligand concentration in the presence of increasing concentrations of an unlabelled competitor (Protocol 1.3).

The concentration of the unlabelled ligand that causes 50% inhibition of radioligand binding is termed the $IC_{50}$. However, this does not necessarily mean that this concentration of ligand will bind half the receptors, as the $IC_{50}$ of the unlabelled ligand will depend upon the ability of the radioligand to bind to the receptor (e.g. its equilibrium dissociation constant), the ability of the unlabelled ligand to bind to the receptor and the concentration of the radiolabelled ligand. Thus, the $IC_{50}$ will normally be greater than the equilibrium dissociation constant $K_b$ of the unlabelled ligand. However, the Cheng–Prusoff equation (Equation 1.4) can be used to determine the $K_b$ of the competitor in a competition binding experiment.

If one of the ligands being used for the assay is an agonist, then it may be desirable to eliminate, or at least minimize, the ability of the G protein to couple to the receptor, so that the interaction between the ligand and the receptor is largely independent of G protein coupling. For this reason, many researchers choose to perform agonist

competition binding assays in the presence of guanine nucleotides and, hence, enable the data to be fitted to a simple one-site binding model. However, if the Hill coefficient of the inhibition curve is significantly different from unity, then this may indicate that the agonist binds to two different receptor species, which are usually described as a high- and low-affinity state. Under these circumstances, binding of the competitor to two sites may be the preferred model (see Equation 1.14).

An alternative approach to competition binding assays measures the competition between the radioligand and a non-radiolabelled version of the radioligand. This type of competition assay is called homologous competition binding [17]. It can be utilized to determine the affinity of the radioligand for a receptor in addition to the number of binding sites present, if the radioligand, A, and unlabelled competitor, B, share an identical affinity for the receptor. Under these circumstances, binding of the radioligand will be a fraction of the total ligand bound ([A] + [B]) and will be defined by

$$[\mathrm{AR}] = \frac{[\mathrm{A}] \times [\mathrm{R_t}]}{[\mathrm{A}] + [\mathrm{B}] + K_\mathrm{a}} \tag{1.11}$$

where

$$K_\mathrm{a} = \mathrm{IC}_{50} - [\mathrm{A}] \tag{1.12}$$

If we consider a heterologous competition binding assay, binding of a radioligand, expressed on the *y*-axis, in the presence of increasing concentrations of a competitor, expressed on the *x*-axis, will follow a sigmoidal curve that can be described by Equation 1.3 (see Figure 1.4). Depending on the nonlinear regression program used to analyse the data, the program must be told to define parameters in a logarithmic

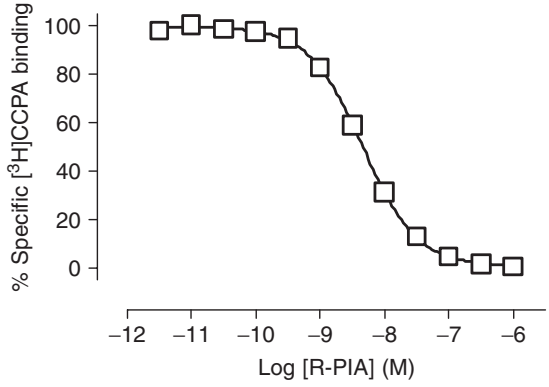


**Figure 1.4** Competition binding of the radiolabelled agonist, [$^3$H]2-chloro-$N^6$-cyclopentyladenosine ([$^3$H]CCPA), at the $A_1$ adenosine receptor by increasing concentrations of the unlabelled agonist, $N^6$-((*R*)-2-phenylisopropyl)adenosine (R-PIA). Membranes were incubated with [$^3$H]CCPA and R-PIA for 1 h at 30 °C before termination of the assay as described in the methods section. Data were transformed to the percentage of specific [$^3$H]CCPA binding and fitted closely to a model that described one-site binding with a Hill coefficient of unity.