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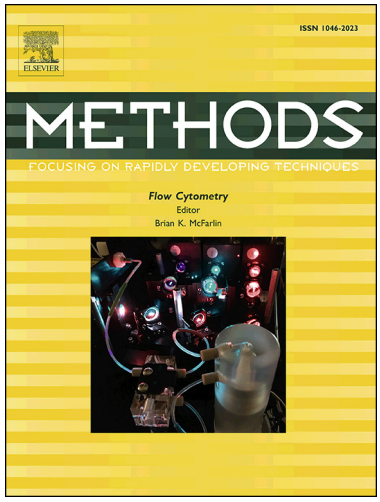

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Functional characterisation of G protein-coupled receptors

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Summary
Characterisation of receptors can involve either assessment of their ability to bind ligands or measure receptor activation as a result of agonist or inverse agonist interactions. This review focuses on G protein-coupled receptors (GPCRs), examining techniques that can be applied to both receptors in membranes and after solubilisation. Radioligand binding remains a widely used technique, although there is increasing use of fluorescent ligands. These can be used in a variety of experimental designs, either directly monitoring ligand itself with techniques such as fluorescence polarisation or indirectly via resonance energy transfer (fluorescence/Forster resonance energy transfer, FRET and bioluminescence resonance energy transfer, BRET). Label free techniques such as isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) are also increasingly being used. For GPCRs, the main measure of receptor activation is to investigate the association of the G protein with the receptor. The chief assay measures the receptor-stimulated binding of GTP or a suitable analogue to the receptor. The direct association of the G protein with the receptor has been investigated via resonance energy techniques. These have also been used to measure ligand-induced conformational changes within the receptor; a variety of experimental techniques are available to incorporate suitable donors and acceptors within the receptor.

1. Introduction

The drive for improved methods of membrane protein isolation comes from the desire to preserve as much of their functionality as possible. This has led to the introduction of new detergents, methods that remove detergent immediately after solubilisation and completely detergent-free protocols [1]. An obvious corollary of these developments is that there are robust methods for measuring receptor function before and after isolation. Of perhaps even greater importance is the need to ensure that a genetically modified receptor retains appropriate properties; the introduction of a point mutation or an epitope tag can seriously impair its function. The techniques can be divided into two broad approaches. One is to examine ligand binding, the other is to measure the ability of the receptor to signal. The latter is generally a more complete measure of functionality, but becomes more demanding when the receptor is removed from the cell.

There are a multitude of techniques available for measuring receptor activity in cells and membranes, prior to solubilisation. A full review of these is beyond the scope of the current article; they are dealt with by others [2-4]. This article will focus on techniques that can be used to measure function in receptors both in membranes and after solubilisation. The latter is much more demanding than the former. For some receptors such as ligand-gated ion channels, any assay of function almost inevitably requires the receptor to be in a lipid bilayer and thus requires reconstitution. However, for other receptors, it is possible to carry out assays when they are still in solution. In this review, we will concentrate on techniques that have been developed for G protein-coupled receptors (GPCRs[1]); variants of many of these can be applied to other receptors with enzymic activity such as tyrosine kinase receptors.

2. Binding assays

[1] Abbreviations: bioluminescence resonance energy transfer, BRET; fluorescence/Forster resonance energy transfer, FRET; G protein-coupled receptors, GPCRs; isothermal titration calorimetry, ITC; surface plasmon resonance, SPR.

2.1. Theoretical perspectives

The binding of a ligand to receptor is usually described by the law of mass action [5]. At equilibrium, the rate at which the ligand, L, binds to the receptor, R, governed by the association rate constant, kon, is equal to the rate at which the ligand-receptor complex, LR, dissociates back to L and R, governed by the dissociation rate constant, koff. Thus:

[L][R]kon = [LR]koff (1),

so [L][R]/[LR] = koff/kon =Kd, the dissociation constant for the ligand at the receptor (2).

If the total number of receptors for L is Bmax and b is the number occupied when it is present at concentration [L], then

$b = Bmax[L]^n/([L]^n + Kd)$ (3), where n is the Hill coefficient; this is a form of the Hill equation.

When n=1, this is consistent with a 1:1 interaction between ligand and receptor. When n>1, the results are conventionally interpreted in terms of positive co-operativity, implying the receptor has multiple binding sites for the ligand and the binding of the first ligand molecule makes it easier for the second to bind. When n<1, there may be negative co-operativity or the the ligand may be binding to more than one type of receptor or binding site.

Equation 3 can be usefully simplified to give the fractional occupancy of the receptor, b/Bmax, simply by dividing both sides by Bmax [6]. It is also usually reformulated for Log[L] rather than [L]. This, the "logistic Hill" equation, gives a sigmoidal curve where n describes the slope of the linear part of the line. The upper asymptote is equal to the Bmax and the Kd is the concentration of ligand needed to occupy half the receptors. However, there will almost certainly be some non-specific binding associated with the ligand. Non-specific binding can be to other receptors in the preparation, reflecting poor selectivity of the ligand but it can also arise from low affinity interactions with the membranes (or detergent micelles) or filters and microfuge tubes that are integral to the assay. It increases linearly with the concentration of ligand and this needs to considered when the total binding of the receptor is measured. Non-specific binding is assessed by conducting parallel assays. In one set, the total binding of the ligand is measured. In the other set, the specific binding of the ligand to the receptor is blocked by inclusion of another agent that saturates all the receptors, leaving only the non-specific binding. Ideally this agent should be structurally dissimilar to the labelled ligand, although that is not always possible [6, 7].

The most straightforward experimental design is saturation binding, where the receptor is incubated with increasing amounts of labelled ligand. This gives a binding curve that should saturate as the Bmax is approached. Importantly, any assay based on this design will give information on the number of receptors in a preparation, as well as their affinity. However, it is not always possible to do this, due to affinity limitations of the ligand leading to the requirement for impractically high concentrations (see below). If a satisfactory asymptote cannot be measured, it will be impossible to measure the Kd with any confidence as this is the concentration needed to half saturate the receptor. In this case, self-displacement of the ligand is used. The concentration of radioligand is kept constant but increasing concentrations of unlabelled ligand are added. Effectively, the specific activity of the radioligand is systematically reduced by dilution with non-labelled ligand. This is a special case of the displacement method, used for characterising the affinity of unlabelled ligands by determining the concentration needed to displace half the labelled ligand (the IC_{50}). The IC_{50} is not the same as the affinity (Ki) for the displacer ligand, as it will be influenced by both the

concentration of the radioligand (L*) and its Kd (Kd*). The Ki can be found by means of the Cheong Prusoff equation

$Ki = IC_{50}/(1 + L^*/Kd^*)$ (4).

If the displacer is really identical to the labelled ligand, then equation 4 reduces to Ki =IC50/2. However, for iodinated ligands, the main situation where the technique is used, this is not necessarily the case. Peptides modified with non-radioactive iodine have been used as displacers to overcome this; more usually, the problem is simply acknowledged in the paper (or ignored!). It is also very difficult to estimate a Bmax with this technique; it relies not only on measuring the Kd (with its associated error) but also measuring very low amounts of ligand bound as the receptor approaches saturation [6, 7].

The Logistic-Hill equation is the basis for all fitting of binding curves. This equation is modified to take account of non-specific binding and can be extended to include more than one specific binding site. It can also be adapted to estimate an IC_{50} or pIC_{50} (ie $-\log IC_{50}$) for displacement experiments. The experimental data is fitted using a suitable non-linear regression method. A variety of software packages are available; perhaps the most widely used are the PRISM family from GraphPad. Typically the data would first be fitted to a four parameter equation that gives estimates of minimum binding, Bmax, Kd/pKd and n. On the basis of n and the overall goodness of fit, it might then be deemed more appropriate to fit the data to a two site model; the fits can be compared by means of an F-test. The binding curves from each experiment should be individually fitted and the means and standard errors calculated from these values. It should be noted the pKd is to be preferred over the Kd for statistical analysis, as only estimates of the former are normally distributed and so can be used with parametric statistics [6, 7].

2.2 Radioligand binding assays

Radioligand binding assays were largely developed in the 1970s, but remain an important tool for receptor characterisation. They can be equally applied to purified receptors and receptors in membranes. In this section, the binding of [^{125}I]-CGRP to its receptors expressed in L6 myocytes is given as an example [8], but the technique can be readily applied to many other GPCRs.

2.2.1 Ligand selection and design

The ligand needs to have adequate specificity for the receptor under consideration. For a purified receptor this is much less of an issue than for one in a native cell membrane, where other receptors may be present. However, even here, non-specific binding to detergent micelles remains an issue. Hydrophobic ligands can partition into micelles; charged ligands will form coulombic interactions with polar or ionic groups on the solubilising agent. It may be possible to mitigate this by increasing the ionic strength of the buffer used for the binding assay; these frequently contain 100 mM NaCl or a similar salt and it is possible to use higher concentrations, although eventually this may impair binding to the receptor itself. It should also be noted that Na^+ is an allosteric regulator at GPCRs. To reduce non-specific binding of a ligand to surface, it can be coated with silane or between 0.1 and 1% bovine serum albumin can be included in the binding buffer [8].

The ligand must be intrinsically of high affinity, with a Kd typically of around 1nM. Much below this and excessive amounts of ligand need to be used. This becomes impractical both in terms of cost

and safety. Furthermore, the greater the concentration of ligand, the larger will be the associated non-specific binding. There are also issues relating to ligand kinetics [6]. A low affinity will invariably mean that the ligand has a rapid dissociation rate. Most methods for separating bound from free ligand result in period when the receptor-ligand complex is isolated from free ligand but not yet captured for assay (for example, during the wash phases on a filter). A rapidly dissociating ligand may be almost entirely lost during this time. Ultra-high affinity ligands bring their own problems. They may be associated with slow and complex kinetics of association. They are particularly difficult to use during in competition assays, where the radioligand is present as a tracer to monitor the binding of a non-labelled compound. The danger is that if the radioligand binds is a pseudo-irreversible manner, a true equilibrium with the displacer is never set up. If the ligand is simply needed as a marker of active receptor during solubilisation and purification, then pre-labelling with a high-affinity, slowly dissociating compound is often advantageous. It may also be possible to modulate ligand affinity, either by the ionic strength, pH and composition of the binding buffer, or by use of a positive or negative allosteric modulator of the binding, assuming such agents exist [7].

Modification of the ligand should not significantly impair its properties. The most common modifications are substitution of ^{3}H or introduction of ^{125}I. The production of a ^{3}H labelled compound, where there has been simple substitution of a ^{1}H atom, will not change the chemical properties of the ligand but production of such compounds usually requires access to specialised chemical facilities. ^{125}I is usually introduced as a substitution onto the aromatic ring of tyrosine in peptides. The iodine atom introduces considerable extra bulk, which may result in steric hindrance or the formation of new van der Waal contacts with the receptor [9]. Histidine can also be iodinated but the same caveats apply. If no suitable endogenous amino acid is present, then an N-terminal tyrosine can be introduced or the peptide modified by derivatisation with the Bolton-Hunter reagent [10]. In these cases, new peptides are produced and clearly they may show different properties from the starting compounds. Iodination can readily be performed in-house by the use of iodogen or other reagents but hplc purification is usually essential to purify mono-iodinated ligands. It should be noted that many commercially available iodinated peptides are principally used in radio-immunoassays, where structural requirements may be less stringent than in receptor binding. The experimenter would do well not to assume a commercially available radioligand will be suitable for receptor binding, unless a stringent analysis has been reported in the literature, including a satisfactory measurement of Kd [7]. Thus, for the peptide adrenomedullin, the C-terminal tyrosine can be iodinated [9], but this residue is now known to be crucial for binding to its receptor [50].

2.2.2 Experimental strategy

The design of a ligand binding assay is usually straightforward. The preparation containing the receptor is first mixed with any unlabelled drug, either to define non-specific binding or for use in a displacement assay and the incubation started by adding the labelled ligand. After sufficient time has elapsed to allow equilibrium to be reached, the bound and free ligands are separated and the fraction containing the bound ligand is analysed by scintillation counting, or whatever technique is appropriate to allow its quantification [6]. There are a number of important practical points that need to be considered.

An appropriate buffer must be used [11]. The effects of the buffer composition on non-specific binding have already been noted. For GPCRs, it may be advantageous to include Mg^{2+} at low millimolar concentration, to promote G protein coupling to the receptor. Particularly if a peptide ligand is used, appropriate peptidase inhibitors may be included. Temperature will of course influence the kinetics of binding; the higher the temperature the faster equilibrium will be achieved but the binding may be less stable due to unfolding or degradation of either the receptor or ligand.

The experience of the authors is that a buffer of 20mM Hepes, 1mM $MgCl_2$, ph7.4, supplemented with 0.1% bovine serum albumin if a hydrophobic ligand is being used, is a good place to start, if no clues are available in the literature [8]. The authors normally conduct binding experiments at rom temperature (25C). Prior to solubilisation, a decision needs to be taken if binding will be measured in intact cells or a membrane preparation. For biochemical studies, membranes are usually to be preferred as they allow greater control of the experimental conditions; particularly useful if the G protein coupled state of the receptor needs to be studied (see section 3.1 for how G-protein association can be manipulated and also reference 11 for an application of this to the study of muscarinic receptors). An important practical point is that the membranes must be well dispersed, to give a homogeneous suspension; this can be achieved by use three, 15 second bursts of a polytron (e.g. Ultra Turrax T25).

It is essential that enough time is allowed for the ligand to reach equilibrium. In competition assays, the time needed for equilibrium to be reached between the labelled ligand and the displacer needs to be considered. It may be possible to establish the time needed for an assay by reference to the literature, otherwise some preliminary experiments will be needed to discover this [5].

A further important issue is that of depletion of labelled ligand. It is normally assumed that the concentration of this does not change during the experiment. However, if the receptor is present in high amounts (as can happen with concentrated, purified receptor), then it will act as sink for the ligand, reducing its concentration throughout the course of the incubation. Depletion should be kept to less than 20%, by reducing the amount of receptor [6, 7].

A variety of methods are available to separate bound from free ligand. For membranes, the two most widely used are centrifugation or filtration. In the former, tubes containing the membrane preparation are spun in a micro-centrifuge (preferentially one that can be cooled) to pellet the membranes. The supernatant is discarded and the tubes washed twice with water (taking care not to dislodge the pellets) before being allowed to dry. If a ^{3}H-labelled radioligand has been used, the pellet needs to be dissolved in a tissue solubiliser prior to scintillation counting. The membrane protein concentration should be around 0.1-0.5 mg/ml. For filtration, the membranes are retained on filters held on vacuum manifolds (cell harvesters); they are rapidly washed two or three times with ice-cold buffer. Lower membrane protein concentrations can be used than with centrifugation assays, but the filters may need to be pre-coated with polyethyleneimine to prevent non-specific binding of the ligand [6, 7].

For soluble receptors, the main assays are either membrane filtration or gel filtration. In the former case, the filters are pre-treated with an agent such as polyethyleneimine, so that the receptor but not the ligand will stick. Gel filtration is of more general applicability; small 1ml columns containing a G10 or G25 resin can either be prepared in the laboratory or purchased. They are spun at low speed in a refrigerated microcentrifuge to ensure that the receptor with bound ligand passes rapidly through, to prevent dissociation [12]. For the solubilised adenosine 2a receptor, BioRad P30 mini-spin gel filtration columns spun at 1000 ***g***, for 4 minutes are a very effective way of measuring the binding of [^{3}H] ZM241385 to the receptor [12].

In principle a scintillation proximity assay can be used, where the scintillant is coupled to the receptor. With this method, there is no need to separate bound from free ligand, as only the bound ligand will cause scintillation (ie it is a homogeneous assay). In practice this assay design is rarely used due to the difficulty of attaching the scintillant to the receptor [13].

2.3 Fluorescence-based assays.

There is increasing use of fluorescent rather than radiolabelled ligands [14]. This is on grounds of safety and convenience, a particular issue where relatively short-lived isotopes are used such as ^{125}I, with a half-life of 60 days. The introduction of a fluorophore into a molecule will inevitable change its chemical structure. For a peptide, it may be possible to conjugate a probe to an existing lysine or cysteine; alternatively a suitable unnatural amino acid can be introduced during synthesis. For non-peptides, more extensive chemical modification is likely to be needed, with the introduction of both fluorophore and a linker. Careful characterisation will be needed to ensure the new molecule still has appropriate pharmacology properties (affinity, specificity, efficacy). The fluorophore itself needs a high quantum yield to allow detection when attached to a ligand at nM concentrations; Alexa Fluor-488, BODIPY and TAMRA are commonly used [15]. Fluorescent ligands can be used just as radioligands in heterogeneous assays where the bound and free ligands are separated. However, there is now much interest in homogeneous assays, where there is no need for this step.

2.3.1 Fluorescent polarisation (FP)

The principle behind this assay is that a ligand in solution will tumble freely and rapidly in all directions. By contrast, when it is bound to large receptor, its movements will be much more restricted. The polarisation of a fluorophore is inversely proportional to its rotation; if this is impeded, then polarisation of fluorescence is increased. The degree of polarisation is independent of the concentration of the fluorophore although the technique assumes that the intrinsic fluorescence of the ligand is not altered by binding to the receptor (if this is the case, then this may itself be the basis of the assay) [16]. There are some limitations of FP. Receptor expression must normally exceed 1pmol/mg and if the receptor is still in membranes or cells, these may settle and disrupt the readings. It requires a high affinity interaction between receptor and ligand, similar to that of radioligand binding. The need for high amounts of receptor relative to ligand means that ligand depletion is often an issue. None-the-less, given an appropriate ligand, the technique can work on membranes as well as for solubilised receptors [16-18]. For the adenosine 2a receptor, Alexa-488, conjugated to the antagonist SCH442416 matched conventional radioligands in membrane binding assays [51]. However, development of the ligand required the use of synthetic organic chemistry, a technique that may not be readily available in many laboratories.

2.3.2 NanoBRET

Bioluminescence resonance energy transfer (BRET) relies on resonance energy transfer between a bioluminescent energy donor (generated by a luciferase) and a fluorescent acceptor which are in close proximity. As the excitation signal is generated by the luciferase, there is no need for an incoming light signal (with attendant problems such as photobleaching) which can limit fluorescence resonance energy transfer (FRET). In Nanobret, a small luciferase, NanoLuc, is fused to the N-terminus of the receptor. This gives a very bright light signal, which is used to excite a bound fluorescent ligand. This is a homogeneous assay with very little non-specific binding, so can be used to measure both high and low affinity interactions in membrane bound and soluble receptors [19]. However, it needs an engineered receptor and a suitable fluorescent ligand. The method has been successfully applied to both the β1 and β2 adrenoreceptors and the A3 adenosine receptor [19]. The limitations are the need to synthesise a suitable fluorescent ligand and also the requirement to engineer the receptor with the N-terminal fusion of the Nanoluc. Thus the technique cannot be used on native receptors and the construct may show different binding properties to the unmodified receptor.

2.4 Label free techniques

There are a number of techniques which do not need the ligand to be modified to allow measurement of its binding. They do however usually require the protein to have been purified and so cannot be used to characterise it in its original state in membranes. Two of the commonest techniques will be considered in this section. They work best when the ligand and receptor are of similar size; thus they are most frequently used to examine the binding of a peptide to the extracellular domain of a receptor (see below). It may be difficult to use this for a comparison with the intact receptor; more usually, relative potencies are be compared rather than absolute affinities. They can be used to measure low-affinity interactions that cannot be investigated by radioligand binding.

2.4.1 Isothermal titration calorimetry (ITC)

ITC measures the heat released or taken up when two molecules associate [20] and is often used to characterise purified proteins. An IT calorimeter has a reaction cell, containing the receptor in solution and a reference cell containing just buffer. Increasing volumes of ligand are titrated into the experimental cell and the energy needed to keep this at the same temperature as the reference cell is measured. From this, for each addition of ligand (a known number of moles), the total energy supplied (the enthalpy, ΔH) can be calculated. A plot of ΔH against the molar ratio of ligand to receptor gives a sigmoidal curve. The amplitude is the enthalpy of the reaction, the molar ratio at the mid-point of the curve gives the stoichiometry and the corresponding slope is 1/Kd [20, 21]. The technique is not particularly sensitive and so may require a substantial quantity of purified and solubilised receptor. On the other hand, the receptor does not need to be modified or attached to a surface. ITC has only rarely been applied to intact GPCRs [22], but it has been used successfully to characterise the binding of ligands to GPCR extracellular domains such as the peptides adrenomedullin and calcitonin to their receptors [23, 24]. For more detailed pharmacological analysis, the authors have synthesised fluorescent derivatives to allow fluorescent polarisation assays.

2.4.2 Surface plasmon resonance (SPR)

SPR measures the change in molecular weight in a target molecule immobilised to an electrically-conducting surface on ligand binding. Polarised light is shone at the surface and this creates plasmons; waves of electron charge density. The polarised light is reflected back from the surface. The plasmons change the refractive index of the surface in proportion to the mass and concentration of the complex. Thus it is possible to monitor the extent and time course of the binding in real time. It is possible to use very small volumes in the flow cells [25]. The receptor does have to be immobilised to a surface (the chip, in SPR terminology) and this involves the engineering of a tag, such as multiple histidine residues or a short peptide sequence which can be recognised by an appropriate antibody which has been coated onto a chip [26]. The purified adenosine 2a receptor has been studied by SPR in detergent, in nanodiscs, in reconstituted lipid membranes or in high-density lipoprotein particles [26, 27]. SPR is an increasingly used technique to examine directly the binding affinity of low-affinity ligands in drug discovery [28] as well as to probe fundamental aspects of GPCR activation [29].

3. Assays of receptor activation

A receptor, as understood pharmacologically, not only binds a ligand; if this is an agonist, it responds to influence the activity of the cell. Thus it is necessary not just to look at the initial binding event but subsequent downstream changes. If the receptor is studied in an intact cell or even in membranes, then a whole range of signalling events can be monitored. For GPCRs, these will be measurements of

second messenger activation, reflecting G-protein coupling, or arrestin coupling, either measured directly or, less certainly, by a downstream signalling pathway. For a solubilised receptor, options are much more restricted. The most common assay is of G protein activation, but other methods are available to investigate changes in receptor state and agonist binding may itself be a measure of receptor function. When a receptor is bound to G protein, an agonist typically shows a higher affinity at than when the receptor is uncoupled [8] (see 3.1. below).

Any assay of receptor activation gives two basic measurements; the potency of the agonist (EC_{50} or pEC_{50}) and the maximum response (Emax). Underlying these is the ability of the agonist to bind to the receptor (affinity) and its ability to activate the receptor (efficacy). A full agonist is one which fully activates the receptor to which it binds; a partial agonist causes incomplete activation. It is now recognised that receptors may have more than one active state and these can differentiate between different signalling pathways. Furthermore, agonists may be able to preferentially activate one pathway over another. In such cases the agonist is described as showing bias; thus one agent may preferentially promote G protein coupling rather than recruitment of arrestin. A number of reviews are available that consider how to quantify efficacy and bias, but the most basic step is to fit the data (in the form of a concentration-response curve) to a logistic-Hill equation of a similar form to that used for ligand binding, to obtain the basal and Emax values, the pEC_{50} and the Hill coefficient. Any software that will analyse a binding curve will also fit a concentration-response curve [7]. It should be noted that the Emax is not the same as the Bmax obtained in binding assays; the number cannot be simply interpreted in terms of the number of receptors present. For the latter, a binding assay is needed.

3.1 G protein association

The simplest conceptional model of agonist action is that it first binds to the receptor and there is then a conformational change, leading to activation; this corresponds to conformational induction. The alternative is conformational selection, where the receptor spontaneously exists in active and inactive forms and the agonist preferentially binds to the active form and so, by mass action, moves the bulk of the population to this state. In either model, the observed Kd for the agonist reflects the affinity for the active form of the receptor. Similar considerations apply to the binding of an inverse agonist, except in this case the ligand preferentially binds to the inactive form of the receptor [7]. For GPCRs, agonists promote a number of conformational changes within the receptor which create a binding pocket for the G protein (or arrestin) and give a receptor with a high affinity for the agonist. G protein association can be disrupted either by GTP or a stable analogue such as GppNHp [7, 8, 34]. Thus in simple terms, if agonist affinity is reduced by addition of 10-100μM GppNHp, then this is good evidence that the receptor can couple to a G protein. This can be applied to receptors in membranes or solubilised receptor G-protein complexes. Evidence for G protein coupling be found from either the direct binding of the agonist if it is labelled, or from the ability of the agonist to displace a labelled antagonist. In the latter case, agonist displacement curves typically show two components (reflected in the first instance by the curve having a low Hill coefficient) reflecting G protein coupled and uncoupled populations of the receptor. Addition of GTP or an analogue should reduce the amount of the high affinity component. The addition of GTP is important to demonstrate that the multiphasic agonist displacement curve is really due to G protein coupling and not binding by the agonist to other receptors [31]. In more recent applications, the presence of GTP (analogue)-shift to an agonist can be used to show the functionality of a receptor engineered for crystallisation [52].

For purified receptors, coupling to individual G proteins can be measured by adding each back in reconstitution experiments [32]. If the assay is performed on membranes made from cells, then the coupling cannot be controlled and will reflect the preference of the receptor in that particular environment.

3.2 Other allosteric modulators

The G-protein is a positive allosteric modulator (PAM) of agonist binding. Measuring this is of especial value as it is part of one of the main physiological pathways that results from agonist binding. However, there are numerous sites at which both positive and negative exogenous allosteric modulators of ligand binding can bind, ranging from the ligand entry site on the extracellular surface of the receptor to its cytoplasmic face. Their effects can most sensitively be detected by effects on receptor kinetics; thus a PAM will slow off rates and a negative modulator will speed this up. If a purified or engineered receptor responds to allosteric modulators in the same way as when in its native membrane in the wild-type state, then this is good evidence that its functional properties are not impaired [33]. As detection of allosteric modulators involves binding assays, they can be used both on membrane-bound and purified receptors; the limiting factor is the availability of an allosteric ligand.

3.3 G protein activation

3.3.1 GTPγS binding

G protein activation may be simply assayed be measuring the association of GTPγS to the receptor-G protein complex. This reflects the physiological process of GDP/GTP exchange that is promoted by an activated receptor [34]. The assay actually reflects the rate of exchange; the active receptor acts catalytically to increase turnover at the guanine nucleotide binding site and so it is important an appropriate time is selected and that all assays use this as near exactly as possible [32]. The time chosen, typically between 5 and 60 minutes, will depend on the G protein (exchange at Gs is slow) as well as the receptor. The assay itself is a simple binding assay (thus applicable to membrane bound and purified receptors), normally with ^{35}S-GTPγS as the ligand, although fluorescent versions have also been used; the fluorescence of BODIPY-FL-GTPγS increases on binding to G proteins and so this has been used in homogeneous assays for the reconstituted parathyroid receptor without the need to separate bound from free ligand [35]. The assay buffer needs to contain around 100nM GDP to suppress non-specific binding. The optimum conditions for the assay may either be established from the literature or by pilot experiments. If the assay is carried out in membranes, then it will reflect the activity of all G proteins that are present and to which the receptor can couple. Coupling to individual G proteins can be established if the membranes are first solubilised and individual G proteins are then immunoprecipated using Protein-A sepharose beads and G protein antibodies with appropriate specificity; critical reading of the literature may be needed to select appropriate reagents [36].

It is possible to directly measure the binding of a purified G protein to a receptor by techniques such as SPR or microscale thermophoresis [37-39].

3.4 FRET and other spectroscopic assays of receptor conformation

It may be possible to directly measure changes in receptor conformation. One approach is to engineer the receptor so that it has an appropriate reporter for spectroscopic detection, using for example fluorescence or electron spin resonance [40, 41]. The engineering can be done in various ways. Many approaches are based on fluorescent reporters. Either changes in properties of the

fluorophore itself may be measured as the receptor changes conformation, or a FRET signal is detected from a second fluorophore that is in close proximity to the first one [40, 42]. Bimolecular FRET is where the donor is attached to one molecule with the acceptor on a second. This method has been applied to GPCRs and arrestins to allow measurement of their association [43]. In unimolecular FRET they are both on the same protein, to allow measurement of intramolecular conformational changes. If luciferase rather than a fluorescent donor is introduced, then a BRET signal can be measured. For GPCRs, a suitably engineered member of the Green Fluorescence Protein (GFP) family is usually attached to the C-terminus. For unimolecular FRET, a second fluorophore needs to be introduced within the sequence in such a way that it does not compromise receptor function. One approach is the introduction of a tetra-cys motif (CCPGCC) which reacts with arsenic-containing fluorophores [44]. This was pioneered with the adenosine 2a receptor. However, it is clear that considerable development work needs to be done to ensure that the tag is introduced at a location that does not impair the function of the receptor. Rather than introduce a tag, it may be possible to introduce a single reactive residue such as a cysteine which can react with a probe to label the receptor. In this case, careful consideration needs to be given to ensuring that only the introduced cysteine reacts with the reporter; most receptors will contain a number of free cysteines [40]. Some of these may be inaccessible to the label, particularly if they are in the transmembrane region; it may be necessary to substitute others with alanine or serine. To avoid these problems, some workers have used unnatural amino acid mutagenesis to directly introduce novel fluorescent amino acids or amino acids that can be derivatised under conditions where no other residue is modified [45]. In all cases, considerable work is needed to engineer a receptor that retains an acceptable wild-type-like phenotype but which can also be used to monitor activity. All FRET-based approaches require engineered receptors.

3.5 Other methods to study receptor conformation.

Apart from spectroscopic labels, receptors can also be engineered with cysteines for use with saturation cysteine scanning mutagenesis (SCAM), where cysteines are systematically introduced and their reactivity to a derivatising agent is monitored [46], or for their ability to form disulphide bonds which only occur after receptor activation (revealed by peptide mapping after protease digestion) [47]. Again, the production and evaluation of these constructs is not a trivial undertaking.

If the protein has been purified, then monitoring changes in overall properties such as endogenous fluorescence may give a measure of receptor activation [48]. However, it is often hard to interpret these signals mechanistically as they reflect contributions from a number of different residues or regions within the protein. Sensitivity to SDS and proteases have also been used as ways of monitoring changes in conformation accompanying receptor activation [49].

4 Conclusions

There are a wide variety of techniques that can be used to study the function of purified GPCRs. Ligand binding lies at the heart of most assays and it is advantageous if the experimenter has a good theoretical grasp of how to analyse ligand-binding curves. Traditionally radioligands were used, but many have been replaced by fluorescent ligands, but the principles behind the assays remain unchanged. Ligand binding can be carried out on unmodified receptors. FRET and BRET-based techniques allow measurement of molecule association and intramolecular distances and these are increasingly applied to study receptor function. They can allow a very sophisticated analysis of receptor function but they need the availability of engineered receptors.

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Appendix; equipment and suppliers

For most of the assays described in this chapter, no specialised equipment is needed beyond that which would be regarded as standard; microfuges, water baths, etc. Likewise, most reagents are available from a range of suppliers. In this appendix some suggestions are provided for more specialised equipment and chemicals; this is not intended to be exhaustive.

Radioligand suppliers

Perkin Elmer (www.perkinelmer.co.uk/category/radiochemicals)

American Radiochemicals (https://arclive.chennova.co.in)

Fluorescent ligand and probe suppliers

Abcam (www.abcam.com/index.html?pageconfig=resource&rid=14885)

Hellobio (https://www.hellobio.com/products/type.html?picat=157)

Thermofisher Scientific (https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/probes-for-endocytosis-receptors-and-ion-channels/probes-for-neurotransmitter-receptors.html)

Tocris (https://www.tocris.com/product-type/fluorescent-probes)

Cell harvesters

These are used for filtration binding assays and are made by numerous suppliers; two examples are given below

Perkin Elmer (http://www.perkinelmer.co.uk/category/cell-harvesters)

Tomtec (www.tomtec.com/cell-harvester.html)

Spin columns

Bio-Rad Microspin columns (http://www.bio-rad.com/en-uk/category/bio-spin-micro-bio-spin-size-exclusion-spin-columns)

FRET and BRET-based assays

Promega supplies suitable constructs for nanoBRET; (https://www.promega.co.uk/products/protein-interactions/live-cell-protein-interactions/nanobret-ppi-starter-systems/?catNum=N1821)

Perkin Elmer supply a variety of kits for detection of second messengers via time-resolved fluorescence, using their LANCE range (www.perkinelmer.co.uk/category/lance-tr-fret)

Thermofisher Scientific supply reagents for FlAsH (https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/cellular-imaging/high-content-screening/flash-and-reash.html)

Isothermal calorimeters

Malvern (https://www.malvern.com/en/products/technology/isothermal-titration-calorimetry)

Surface Plasmon Resonance (SPR)

GE Healthcare supply Biacore instruments and reagents for SPR (http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-uk/products/AlternativeProductStructure_11926/)

Data analysis

Graphpad supply the PRISM software (https://www.graphpad.com/scientific-software/prism)

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Highlights

Theory of ligand binding

Use of radioligands

Use of fluorescent ligands

Label-free techniques for studying receptors

GTP binding to probe G protein activation

BRET and FRET to study receptor activation