Review

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A crystal clear solution for determining G-protein-coupled receptor structures

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G-protein-coupled receptors (GPCRs) are medically important membrane proteins that are targeted by over 30% of small molecule drugs. At the time of writing, 15 unique GPCR structures have been determined, with 77 structures deposited in the PDB database, which offers new opportunities for drug development and for understanding the molecular mechanisms of GPCR activation. Many different factors have contributed to this success, but if there is one single factor that can be singled out as the foundation for producing well-diffracting GPCR crystals, it is the stabilisation of the detergent-solubilised receptor–ligand complex. This review will focus predominantly on one of the successful strategies for the stabilisation of GPCRs, namely the thermostabilisation of GPCRs using systematic mutagenesis coupled with thermostability assays. Structures of thermostabilised GPCRs bound to a wide variety of ligands have been determined, which has led to an understanding of ligand specificity; why some ligands act as agonists as opposed to partial or inverse agonists; and the structural basis for receptor activation.

The challenges of GPCR structure determination

GPCRs make up a diverse family of integral membrane proteins that have a conserved structure composed of seven transmembrane α-helices [1]. Their function is to bind a signalling molecule, typically a hormone or neurotransmitter, which induces a change in receptor conformation that propagates to the cytoplasmic surface. This activated conformation of a GPCR can then bind and activate an intracellular heterotrimeric G protein, resulting in the increase of intracellular second messengers such as Ca^{2+} or cAMP. GPCRs are also capable of activating the mitogen-activated protein kinase (MAPK) pathway, through a G-protein-independent mechanism that requires receptor phosphorylation and recruitment of arrestin [2]. GPCRs are dynamic proteins that are in equilibrium between several conformational states [3]. Two important conformations are the R and R* states, which are defined by an inability (R state) or ability (R* state) to activate G proteins [4]. The key conformational changes upon receptor activation were elucidated by comparison of the structure of a G protein bound to an activated GPCR (R* state [5]) with previous structures of receptors in the R state. However, the changes in receptor structure upon recruitment of arrestin remain undefined, although changes at the receptor C terminus are likely to be involved [6,7]. The pivotal role GPCRs play in intercellular signalling and the regulation of multiple physiological processes makes them key targets for the pharmaceutical industry [8,9]. However, it is only in the past few years that structural biology has begun to elucidate how GPCRs function and to provide a platform for structure-based drug design [10,11].

The first structure of a GPCR to be determined was bovine rhodopsin [12]. Rhodopsins are unique amongst GPCRs in having a covalently bound chromophore that isomerises upon absorption of a photon of light, which induces a conformational change in the receptor [13]. Rhodopsin was the first GPCR to be crystallised [14] because it is naturally abundant in the retina of the eye and it is extremely stable in a detergent-solubilised state, provided that it is kept in the dark [15,16]. This stability is a consequence of rhodopsin having evolved a large activation energy barrier between the R and R* states, to remain in the inactive R state in the dark [17]. By contrast, most other GPCRs have evolved to be exquisitely sensitive and specific sensors for low concentrations of small molecules or ions. This has resulted in many of the receptors having low thermal barriers between the R and R* states, which means that receptors usually exhibit basal activity [18]. That is, receptors can still activate G proteins even in the absence of the activating molecule (agonist; Box 1), although to a lesser extent than in the agonist-bound state, because there is always some receptor in the R* state. This dynamism of GPCRs is one of the reasons why there has been so much difficulty in crystallising non-rhodopsin GPCRs, despite large amounts of some heterologously expressed GPCRs being available for many years before the structure of rhodopsin was determined [19,20]. Although the dynamism of GPCRs is a significant impediment to crystallisation, this can often be overcome by binding high-affinity ligands that lock the receptor in a single conformation.

The other key issue that has hindered crystallisation of GPCRs is the poor stability of the detergent-solubilised receptor in a form that is amenable for crystal formation. GPCRs are often small proteins with a molecular weight of about 35 kDa, and if they are purified in mild detergents such as dodecylmaltoside, then the hydrophilic regions of the protein are often occluded by the detergent micelle [21–23]. These hydrophilic regions are essential to form crystal contacts, therefore, dodecylmaltoside is not a good detergent for crystallising GPCRs by vapour diffusion, but small detergents such as octylglucoside should, in theory, be ideal. Unfortunately, small detergents are often highly denaturing, which precludes their use for the majority of

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Box 1. Pharmacological definitions

Ligands or drugs that interact with GPCRs are defined according to their activity when added to cells that contains the specific GPCR of interest. Each ligand is added to whole cells at a variety of concentrations and a biological response is measured (Figure I), such as the increase in intracellular cAMP or the increased fluorescence of an intracellular Ca^{2+}-responsive dye. Definitions of ligands according to their biological activity are listed below. However, different responses may be measured depending on the cell type used, the number of copies per cell of the receptor and the specific assay used. This may on occasion lead to conflicting definitions in the literature.

Agonist: a ligand that binds to and activates a receptor and elicits a physiological response. The endogenous agonist for the β_1AR, noradrenaline, is a full agonist (Figure I, red line) that elicits the maximal response for the receptor in activating a G protein.

Basal or constitutive activity: a physiological response that occurs in the absence of an agonist due to a proportion of the receptor being in the activated state.

Inverse agonist: a ligand that binds to a receptor and inhibits or eliminates, in the case of a full inverse agonist, the basal or constitutive activity of a receptor (Figure I, green line).

Neutral antagonist: a ligand that neither stimulates receptor activation nor inhibits basal activity (Figure I, black line).

Partial agonist or weak partial agonist: a ligand that elicits only a partial response when compared to a full agonist (Figure I, blue and yellow lines).

Antagonist: any ligand that blocks binding of endogenous agonists to the receptor. This is a general term that encompasses ligands that may be inverse agonists, partial agonists or neutral antagonists.

Biased agonist: a ligand that binds to a receptor and signals to a variable extent through both G-protein-dependent and G-protein-independent pathways.

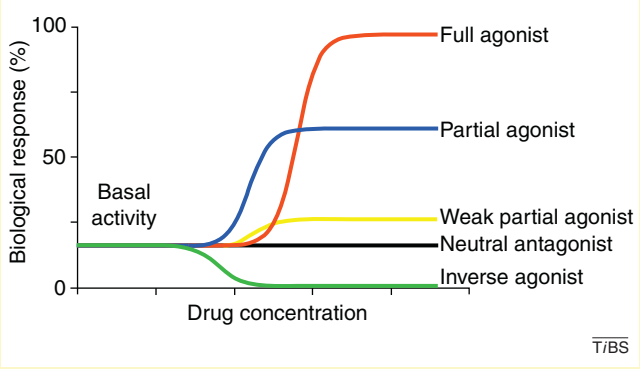

Figure I. Pharmacological effects of different ligands.

GPCRs. This has meant that large detergents have to be used for receptor purification and some receptors have to be further stabilised by the inclusion of cholesteryl hemisuccinate [24,25], which can increase the micelle size even further. Thus, there are two options for promoting crystal formation of GPCRs in detergent micelles: (i) increase the hydrophilic area of the protein by using antibodies or fusion proteins so that a detergent with a large micelle can be used; or (ii) make the membrane protein more tolerant to short-chain detergents, by increasing its thermostability.

Strategies for increasing the hydrophilic area of GPCRs

The strategy of increasing the hydrophilic area of the GPCR (Figure 1) has had a lot of success in determining structures of GPCRs. For instance, the first structure of a non-rhodopsin GPCR [26], the β_2-adrenoceptor (β_2AR), was determined by binding an F_{ab} antibody fragment to cytoplasmic loop 3 (CL3), using a methodology that was originally developed for crystallography of mitochondrial membrane proteins [27]. A high-resolution structure of β_2AR was subsequently determined using a fusion protein approach where T4 lysozyme (T4L) was fused into CL3 to make a β_2AR–T4L fusion protein [28,29]. This methodology, originally developed to determine the topology of bacterial membrane proteins [30], had been attempted previously to crystallise the bacterial transporter LacY [31,32], although high-quality crystals were not obtained. The successful structure determination of β_2AR–T4L was a result of the careful engineering of the junction sites between T4L and β_2AR [29], which was necessary to ensure the chimera was correctly folded and stable, combined with the use of lipidic cubic phase (LCP) as the crystallisation matrix [33,34]; high-quality receptor–T4L crystals have not been grown in detergent by vapour diffusion. Crystallising the receptor while bound to a high-affinity inverse agonist, carazolol, turned out to be crucial for the structure determination of both the β_2AR–F_{ab} complex and β_2AR–T4L. This ligand probably has two effects. First, it locks the receptor in a single conformation (R state) and, second, it stabilises the receptor during crystallisation. The T4L fusion promotes crystal contact formation, but it does not have a significant effect on the thermostability of the receptor and could in theory decrease receptor stability, depending upon the particular receptor and the positions of the fusion points. For this reason, structural determination of GPCR–T4L fusions are usually coupled with binding high-affinity ligands, preferably with a slow rate of dissociation, that impart substantial thermostability to the detergent-solubilised receptor. A significant advance in this general strategy has been to develop proteins that bind to a receptor, lock it in a specific conformation, and reduce the flexibility of loop regions. For example, the single-domain heavy chain camelid antibody fragment, Nb80, and Gs, a heterotrimeric G protein, can both lock β_2AR in the activated R* state, allowing the structure of the activated β_2AR to be determined [5,35]. By contrast, a mouse monoclonal F_{ab} antibody fragment locks the adenosine A_{2A} receptor (A_{2A}R) in the inactive R state, which allows the structure of the A_{2A}R–F_{ab} complex to be determined from crystals grown by vapour diffusion and using large-micelle detergents [36]. One potential advantage of using antibody fragments to crystallise GPCRs is that it might be possible to obtain structures of receptors bound to low-affinity ligands, because the antibody–receptor complex might be more thermostable than the receptor alone, although this has not yet been tested. Currently, the only strategy that has yielded structures of GPCRs bound to low-affinity or endogenous ligands is conformational thermostabilisation [37,38] of the receptor by systematic mutagenesis. This second strategy is the focus of the remainder of the review.