BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 986–996

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: MOLECULAR PHARMACOLOGY OF G PROTEIN-COUPLED RECEPTORS

REVIEW

The impact of GPCR structures on pharmacology and structure-based drug design

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After many years of effort, recent technical breakthroughs have enabled the X-ray crystal structures of three G-protein-coupled receptors (GPCRs) (β1 and β2 adrenergic and adenosine A_{2a}) to be solved in addition to rhodopsin. GPCRs, like other membrane proteins, have lagged behind soluble drug targets such as kinases and proteases in the number of structures available and the level of understanding of these targets and their interaction with drugs. The availability of increasing numbers of structures of GPCRs is set to greatly increase our understanding of some of the key issues in GPCR biology. In particular, what constitutes the different receptor conformations that are involved in signalling and the molecular changes which occur upon receptor activation. How future GPCR structures might alter our views on areas such as agonist-directed signalling and allosteric regulation as well as dimerization is discussed. Knowledge of crystal structures in complex with small molecules will enable techniques in drug discovery and design, which have previously only been applied to soluble targets, to now be used for GPCR targets. These methods include structure-based drug design, virtual screening and fragment screening. This review considers how these methods have been used to address problems in drug discovery for kinase and protease targets and therefore how such methods are likely to impact GPCR drug discovery in the future.
British Journal of Pharmacology (2010) **159,** 986–996; doi:10.1111/j.1476-5381.2009.00476.x; published online 13 November 2009

This article is part of a themed section on Molecular Pharmacology of GPCR. To view the editorial for this themed section visit http://dx.doi.org/10.1111/j.1476-5381.2010.00695.x

Keywords: G-protein-coupled receptor; X-ray crystallography; structure-based drug design; selectivity; conformation; dimerization; allosteric regulators; fragment screening; virtual screening; rhodopsin

Abbreviations: BACE, β-site of APP cleaving enzyme; DPPIV, dipeptidyl peptidase-4; EGFR, epidermal growth factor receptor; FXa, Factor X; GPCR, G-protein-coupled receptor; HDAC, histone deacetylases; HTS, high-throughput screening; NAM, negative allosteric modulator; PAM, positive allosteric modulator; ROCK1, rho-associated; coiled-coil, containing protein kinase 1; SBDD, structure-based drug design; SPR, surface plasmon resonance; TM, transmembrane

Introduction

High-resolution 3-dimensional structures of proteins provide a detailed understanding of the form and function of such proteins at the molecular level. This is useful not only to describe the aspects of protein structure which underlie physiological processes but also in visualizing the intimate connections that bind proteins to their ligands and to small molecule drugs. Although structural studies have been highly successful for soluble proteins, progress in solving the structures of membrane proteins has been relatively poor. G-protein-coupled receptors (GPCRs) represent one of the most important classes of protein due to their critical role in cell signalling in response to hormones and neurotransmitters. GPCRs are the site of action for a wealth of small molecule and biological drugs across many therapeutic areas; however, until recently, the only structure known from this family was that of the visual pigment rhodopsin (Palczewski *et al.*, 2000). Knowledge of how drugs interacted with receptors was limited to models based on homology with rhodopsin or from site-directed mutagenesis experiments. In the last 2 years a number of different technological developments have resulted in the structures of three new GPCRs, all of which are

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Received 24 April 2009; revised 23 June 2009; accepted 5 August 2009

important drug targets; the β1 and β2 adrenergic receptors and the adenosine A_{2a} receptor (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008).

The properties of the new structures and the technological developments which led to them have been reviewed in detail elsewhere (Shukla *et al.*, 2008; Weis and Kobilka, 2008; Hanson and Stevens, 2009). The aim of this review is to examine the impact of GPCR structures on recent theories in the pharmacology and signalling of these receptors, as well as how structure will influence the discovery of new drugs. In particular, comparisons will be drawn from the enzyme field where 3D structures have had a major impact on drug discovery, aiding the design of molecules with improved selectivity and pharmaceutical properties.

Rhodopsin

The overall topology of GPCRs with 7-transmembrane spanning regions was first shown from two-dimensional crystals in 1993 (Schertler *et al.*, 1993). However, it was 7 years until the first high-quality 3-dimensional structure of bovine rhodopsin in the ground state was published (Palczewski *et al.*, 2000). These data revolutionized our understanding of GPCRs and provided a template of sufficient quality to start modelling other members of the family. However, rhodopsin differs from other receptors in that its ligand, 11-*cis* retinal, is covalently linked to the protein (opsin) where it acts as a full inverse agonist to hold the receptor in the inactive conformation (Okada *et al.*, 2001). Upon absorption of light, isomerization of the ligand from the *cis* to *trans* form converts it to a full agonist and leads to activation of the receptor. An important recent development has been the structure of opsin in the absence of ligand, both alone and in combination with a peptide from the G protein transducin (Park *et al.*, 2008; Scheerer *et al.*, 2008). These structures have significant changes in the position of the helices that are consistent with the receptor being in an active or partially active conformation.

Recent developments in obtaining GPCR structures

There have been many technical problems which have prevented the crystallization of GPCRs other than rhodopsin. A key requisite in the formation of the well-ordered crystals required for a high-resolution structure is the availability of stable proteins in a uniform conformation and state. Recent advances in protein expression have enabled the production of large quantities of recombinant GPCRs from bacterial and insect cells (Lundstrom *et al.*, 2006; Mancia and Hendrickson, 2007). GPCRs are inherently flexible due to their requirement to change between conformations during the process of activation. As a result, a population of GPCRs in the absence of ligand will usually encompass receptors across a spectrum of conformations from the inactive ground state (R) through to the fully active R* coupled to G proteins (Kenakin, 2001). This heterogeneity of conformations generally prevents the formation of protein crystals. A second problem in the crystallization of any membrane protein is the fact that the membrane itself provides a significant contribution to stabilizing the folding and maintaining the structure of the protein. When removed from the membrane environment, GPCRs will rapidly denature (Wiener, 2004). Conditions for solubilization and crystallization must be carefully determined in order to maintain functional receptors; however, often these are not consistent with the conditions required for crystallization. For example, receptors usually require long chain detergents to maintain functional conformations, whereas crystallization is most likely to occur in the presence of short chain detergents (Wiener, 2004). The recent structures have overcome these problems in a number of ways – a key component of which was to lock the receptors into a particular conformation. The third intracellular loop (IC3) is a highly flexible region of the receptor involved in coupling to G proteins. Changes in this region have been shown to occur during receptor activation (Ghanouni *et al.*, 2001a,b). In the two structures of β_2AR, this region was constrained by either the binding of a monoclonal antibody (Rasmussen *et al.*, 2007) or insertion of a fusion protein consisting of the small stable cytosolic protein T4 lysozyme (T4L) (Cherezov *et al.*, 2007). The T4L fusion approach was also used in the crystallization of the adenosine A_{2a} receptor (Jaakola *et al.*, 2008). In the case of the β1AR, a different approach was taken (Serrano-Vega *et al.*, 2008; Warne *et al.*, 2008). The protein was stabilized by introducing a limited number of point mutations that were shown to significantly increase the overall thermostability of a particular conformation of the receptor. This conformational thermostabilization was thought to be due to a combination of changes which reduced the flexibility of the protein and that biased the equilibrium towards an antagonist conformation. The effect on conformation could be demonstrated pharmacologically in binding studies with agonists or antagonists. The 'fixing' of the conformation in this way was likely to have had the effect of reducing heterogeneity in solution and encouraging crystallization. An increase in thermostability also resulted in an increased stability in detergents compatible with crystallization.

Impact of structure on understanding GPCR function

Conformation

The ability of GPCRs to take different conformations which differ in affinity for agonist and antagonists ligands is well recognized in pharmacology. However, in the absence of structure, the molecular changes within the helical bundle which underlie these conformations is unknown. A major benefit of the new GPCR structures will be to provide physical evidence of the conformations predicted by receptor theory. In order to define the conformations of receptors observed in crystal structures, it is of vital importance to carefully characterize the conformational state of the protein being crystallized, as defined by the rules of pharmacology. Current models of receptor theory presume that the receptor can exist in at least two major conformations designated as R, the ground or inactive state; and R*, the active state. These states exist in an equilibrium which is shifted by the binding of ligands. Agonist ligands promote formation of the R* state while inverse

agonists promote formation of the R state. In cells, levels of basal activity are determined by the proportion of receptors in the R* state. Shifts between conformational states can usually be determined by measuring the affinity of ligand binding such that agonists have a higher affinity for the R* state compared with the R state. For inverse agonists, the reverse is true. Neutral antagonists bind to both forms with equal affinity. These differences in affinity are not always apparent when carried out on mixed populations of receptor conformations present in membranes due to limitations of the assay; however, they are more easily seen when assaying purified receptors locked in specific conformations prior to crystallization. According to the ternary complex, model the receptor must isomerize to the active state before coupling to G proteins; however, in the cubic ternary complex model, a conformation is defined which couples to G proteins but does not signal (Kenakin, 2004).

Rhodopsin undergoes a series of conformational changes in its transition from inactive rhodopsin to the fully active state, metarhodopsin II. A series of distinct photo-intermediates can be studied due to changes in their absorption of light (Ballesteros and Palczewski, 2001; Palczewski, 2006; Ridge and Palczewski, 2007). Whether all these different states also occur in other GPCRs is not yet clear; however, it seems likely that the major changes in the positions of the helices that occur during activation are likely to be conserved across at least all Family A GPCRs. The E/DRY motif at the end of TM3 is very highly conserved across Family A GPCRs and is thought to be a key region involved in receptor activation. In the dark (ground) state of rhodopsin, a salt bridge occurs from residues in the DRY motif linking TM3 to TM6. This interaction is known as the 'ionic lock'.

The technical problems associated with obtaining crystal structures outlined earlier means that modifications to the wild-type protein have been necessary to obtain high-resolution structures of other GPCRs (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008). In all cases, these modifications have altered the conformational equilibrium of the receptor and indeed this has probably contributed to their crystallization. Insertion of T4L into the IC3 loop of β2AR had the effect of shifting the equilibrium of the receptor into an agonist-like state, such that the affinity of agonists and partial agonists (e.g. isoproterenol and salbutamol) is increased compared with the wild-type receptor. Furthermore, the receptor appears to be in a form analogous to the constitutively active state, as indicated by the signal from a fluorescent probe bound within the receptor that was used to act as a reporter for ligand-induced conformational changes (Rosenbaum *et al.*, 2007). A direct measure of constitutive activity was not possible as insertion of the T4L fusion prevents coupling to G proteins. Under normal conditions, β_2AR shows some basal activation and in these systems the ligand carazalol behaves as a partial inverse agonist, reducing basal activity but not resulting in a fully 'off' receptor (the latter would be analogous to the dark-state of rhodopsin). In the β2AR, the ionic lock is not able to form due to an increased distance between TM3 and TM6 compared with rhodopsin. The adenosine A_{2a} structure also contains a broken ionic lock; however, this structure again included the T4L fusion that resulted in an agonist-like pharmacology – a higher affinity for agonists measured in radioligand binding studies. The A_{2a} receptor was crystallized in the presence of the antagonist ZM241385 (Palmer *et al.*, 1995; Jaakola *et al.*, 2008).

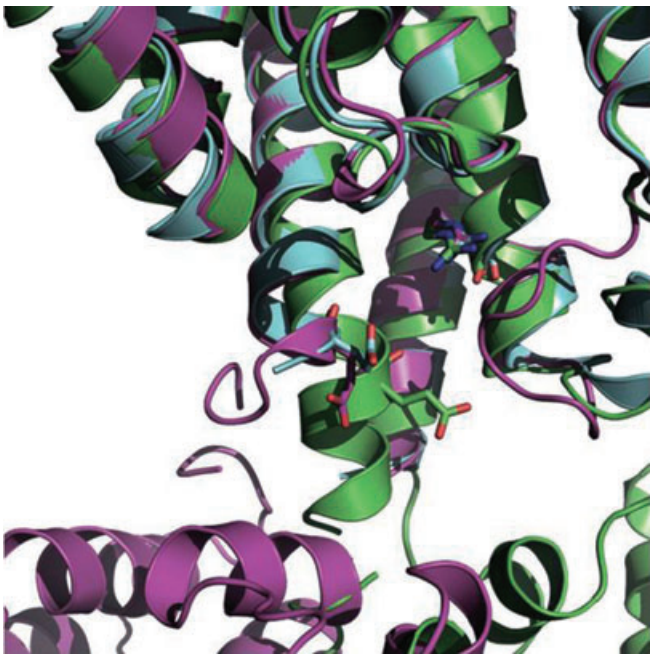

Figure 1 Superimposition of the 'ionic lock' of the β1AR, β2AR and Adenosine A_{2a} crystal structures, illustrating that the salt bridge between the key arginine and glutamate residues in the DRY motif linking TM3 to TM6 are broken in each case. The receptors are believed to be in an 'almost off' state, but it can be seen that each structure has the glutamate in significantly different positions with respect to one another and further structures are required to rationalize these differences. β_1AR in blue, β_2AR in pink, Adenosine A_{2a} in green.

In the case of the β_1AR, mutagenesis resulted in a change of equilibrium of the receptor towards an antagonist conformation. For this receptor, there was a reduction in agonist affinity compared with the wild type; however, the receptor was still able to couple to G protein in the presence of a very high agonist concentration. The crystal structure contained the compound cyanopindolol in complex with the protein. This ligand is generally considered to be an antagonist; however, it can behave as a very weak agonist in well-coupled systems (Brawley *et al.*, 2000). A comparison of all three structures shows close similarity in the positions of the cytoplasmic ends of the helices and in all cases the ionic lock is broken (Figure 1). This suggests that all three receptors are in equivalent conformations that represent antagonist-bound inactive forms. The absence of the ionic lock may be due to differences between rhodopsin and other receptors or perhaps that these receptors are in an 'almost off' state which is not quite the same as the fully off dark state of rhodopsin. Further structures of GPCRs in the presence of full inverse agonists rather than partial agonists or partial inverse agonists should resolve this fascinating question.

Receptor activation and signalling

The ligand bound to rhodopsin (11-*cis* retinal) is a strong inverse agonist which maintains the receptor in the completely inactive dark state. In the absence of any ligand, the

protein opsin can bind and activate G proteins and can be considered a constitutively active receptor (Vogel and Siebert, 2001; Lamb and Pugh, 2004). In 2008, Park *et al.* (2008) were able to crystallize purified opsin and obtained the first structure of an activated GPCR. The main changes associated with this activation were a movement in the ends of TM5-TM7 resulting in a rearrangement of both the second and third cytoplasmic loops. In addition, TM6 moves outward particularly at the cytoplasmic end; perhaps to enable binding of the G protein. In this partially activated receptor, the ionic lock is broken and two new interactions stabilize the ends of TM5 and TM6. There is also a deviation of TM7 which includes another highly conserved region important in receptor activation – the NPxxY motif. More recently, the same group (Scheerer *et al.*, 2008) has crystallized opsin in complex with an 11-amino acid peptide derived from the extreme C-terminus of the transducin $G\alpha_t$ subunit (Gα–CT). The peptide from the G protein binds to a crevice formed by movement of TM5 and TM6 where the carbonyl side chain of Cys347 interacts with Arg135 from the E/DRY motif. Within the receptor, there exists a network of stabilizing interactions which connect the ligand-binding site through to the intracellular ends of the helices. Binding of either agonist within the ligand-binding site or G protein to the intracellular G protein binding region appear to both be able to stabilize the active conformation and increase the affinity for the other. Other studies involving a new version of electron paramagnetic resonance known as double electron-electron resonance also indicated that an outward movement of TM6 and smaller movements of TM1 and TM7 occur on activation (Altenbach *et al.*, 2008; Huber and Sakmar, 2008).

The concept of a receptor existing in a simple pair of active and inactive states (R and R*) is no longer sufficient to explain the observations of pharmacology. Agonists vary considerably in their efficacy and how this relates to the bound conformational states is unclear. A partial agonist with 50% efficacy could fully activate 50% of the receptors or could activate 100% of the receptor by 50%. Alternatively, a partial agonist might stabilize a different form of the receptor to a full agonist state and this different conformation might activate the G protein with a lower efficiency. The study of rhodopsin suggests that activation of the receptor involves the release of key structural constraints within the E/DRY and NPxxY regions. Energy provided by agonist binding must be sufficient to break these constraints and stabilize the new active conformation. In the case of rhodopsin, whether this transition is complete or partial depends on the chemical nature of the ligand (Fritze *et al.*, 2003). The retinal analogue 9-demethylretinal is a partial agonist of rhodopsin which only poorly activates G protein in response to light. Spin-labelling studies (Knierim *et al.*, 2008) suggest that in the presence of this ligand, only a small proportion of receptors are in the active conformation equivalent to all-*trans*-retinal. However, this can also result in a new state that is not formed with the full agonist. Therefore, rhodopsin studies suggest that that partial agonism may result in either a reduced number of fully active receptors or conformations which are not capable of fully engaging the signal transduction process. Structures of other GPCRs in complex with partial agonists are required to determine their effects on conformation.

A recent concept in GPCR pharmacology is that of agonist-specific signalling, also known as stimulus trafficking or biased agonism (Kenakin, 2001; Baker and Hill 2007; Galandrin *et al.*, 2007). It is now clear that binding of different agonist ligands to a single receptor can result in differential activation of signalling pathways within the cell. In addition, the same ligand acting through a single receptor type can be an agonist on one signalling pathway and an antagonist at another within the same cell. This has important implications for drug discovery. The most likely explanation of these effects is that multiple active conformations exist that have different abilities to couple through G proteins or other effectors to different signalling pathways. Indeed, conformations may exist which can only activate a single pathway within a cell and therefore a ligand which stabilizes this conformation will be an agonist for that pathway while being an antagonist at a different pathway that requires an alternative conformation.

Crystal structures in complex with ligands purported to signal through different pathways could provide a molecular explanation for biased agonism. Yet again the β-adrenergic receptors may provide the model system for this. β-adrenergic receptors couple primarily to $G\alpha_s$ to increase cAMP levels; however, under certain circumstances they can also signal through $G\alpha_i$ (Daaka *et al.*, 1997). The latter can result in inhibition of adenylate cyclase and lowering of cAMP via Gα as well as activation of MAP kinase pathways by Gβγ. β-adrenergic receptors can also signal independently of G proteins via β-arrestin coupled signalling. The β-arrestin pathway can result in activation of MAP kinase pathways (Lefkowitz and Shenoy, 2005; DeWire *et al.*, 2007). Recently, a variety of ligands have been identified for both β1 and β2 which can differentiate between these various pathways. In the case of β1, isoproterenol and bucindol are agonists through $G\alpha_s$ while propanolol is an antagonist. However, all three ligands could also activate MAP kinase pathways; isoproterenol activated MAP kinase pathways via $G\alpha_i$, whereas bucindol and propanolol activated MAP kinase signalling independent of G proteins and independent of β-arrestin (Galandrin *et al.*, 2008). For β2 receptors, carvedilol has unusual pharmacology as it is an inverse agonist for $G\alpha_s$ signalling to adenylyl cyclase while being an agonist for β-arrestin recruitment and activation of MAP kinase (ERK1/2) (Wisler *et al.*, 2007).

Dimerization

Many GPCRs are now known to function in cells as homo or heterodimers or higher-order oligomers. Dimerization may be involved in trafficking receptors, ligand binding, cell signalling and desensitization (Milligan, 2007; Gurevich and Gurevich 2008; Ferré *et al.*, 2009). There has been considerable debate with regard to the structure of GPCR dimers and the nature of the dimer interface in the basal and activated state. Structural data on these interfaces is limited and to date, most information had been obtained from a variety of techniques, including modelling, mutagenesis and cross-linking (Hebert *et al.*, 1996; Guo *et al.*, 2005; Fotiadis *et al.*, 2006) which have implicated TMs 1,4,5 and 6 at the boundary between receptor subunits.

Obtaining physiologically relevant structural data from biophysical and crystallography studies is problematical and subject to artefacts which occur as a result of the addition of detergent during solubilization and the propensity for solubilized monomers to assemble in a non-physiological fashion. GPCR dimers normally exist within the plasma membrane and the membrane phospholipids will play a significant role in the stability of the dimer. Upon detergent solubilization, it is likely that all but the most stable dimers will dissociate. Artefacts will also occur during the crystallization process via the formation of crystallization dimers during the packing of protein subunits within the crystal lattice. Contacts made during crystallization may often involve contact surfaces which would not interact in the native membrane. In many cases, GPCRs will form head to tail dimers in crystals which are clearly not physiological. For example, in the first structure of ground-state rhodopsin (Palczewski *et al.*, 2000), head to tail dimers clearly not present in the membrane were seen. The use of the T4 lysozyme fusion protein approach to facilitate crystallization results in crystallographic interfaces between the lysozyme molecules or between lysozyme and the receptor. The nature of these interfaces differed considerably between the β2 and A2a structures (Cherezov *et al.*, 2007; Jaakola *et al.*, 2008).

Dimers of rhodopsin were observed in native rod disc membranes using atomic force microscopy (AFM) and electron microscopy (EM) (Fotiadis *et al.*, 2003; Liang *et al.*, 2003; Fotiadis *et al.*, 2004). In these dimers, TM4 and 5 are the main point of inter-subunit contacts and this fits with the dopamine D2 dimers interface reported by Javitch's group (Guo *et al.*, 2005). Dimers were also observed in 2D crystals of Meta rhodopsin I using cryo-electron microscopy (Ruprecht *et al.*, 2004). In this case, dimers were in the same orientation as in the membrane with the subunit interface mediated between TM1 of each molecule. The short helix formed by the C-terminus known as H8 also forms a close interaction between subunits. Similar dimers are observed in the 2D crystals of bovine and frog rhodopsin (Schertler *et al.*, 1993; Schertler and Hargrave, 1995). Two more recent X-ray crystal structures of bovine rhodopsin also implicate TM1 and H8 as well as TM2 in dimer contact (Lodowski *et al.*, 2007).

Although the availability of increasing numbers of GPCR structures may contribute to our understanding of the structure of GPCR dimers, this information must be interpreted with caution due to the likelihood of crystallization artefacts. Data using other techniques such as mutagenesis and AFM will be needed to verify any observations from 3D structures.

Introduction to structure-based drug design

Structure-based drug design (SBDD) is the process by which information about the way a given ligand binds to its target receptor is used to derive new drugs against that target. Generally, the ligands will be small molecules which might include the endogenous ligand for the system or hits which have been identified from high throughput, virtual or fragment screening (outlined later). In other cases, the ligands may be peptides which are known modulators or substrates of the target. Having information about which parts of the ligand form energetically favourable interactions with the receptor (electrostatic, hydrogen bonding, van der Waals or hydrophobic contacts) and the conformation of the ligand serves to focus the drug design efforts undertaken by medicinal chemists. Visualization of the binding site also highlights areas which are not yet being exploited by the ligand, or indicates that some parts of the ligand may be redundant and might be deleted without cost to binding.

Protein-ligand crystal structure information is the most often used source of data for SBDD. There are now many examples of marketed drugs whose development has been dependent on protein-ligand structures (Congreve *et al.*, 2005; Mittl and Grütter, 2006). Early successes include HIV protease in which a number of drugs including nelfinavir, amprenavir and lopinavir were rapidly developed during the 1990s and neuraminidase for which zanamivir and oseltamivir were launched as treatments for influenza in 1999. Zanamivir was designed *de novo* from structural information of the enzyme's substrate and required little further medicinal chemistry optimization (von Itzstein *et al.*, 1993). In the early part of this century, the development of kinase inhibitors for the treatment of cancers has been a major focus of pharmaceutical companies' endeavours and is driven by protein-ligand crystal structures of multiple chemical series (or chemotypes) in the adenosine triphosphate (ATP) binding site of multiple kinase enzymes. The challenge has been developing agents which are selective for one or more kinases of interest, avoiding activity versus other related kinase enzymes while retaining good pharmaceutical properties. The prototype drug in this field is imatinib (Gleevec) which targets the BCR-ABL kinase and was launched in 2001 for the treatment of chronic myelogenous leukaemia (Zimmermann *et al.*, 1996; 1997). Another anticancer kinase inhibitor drug, erlotinib (Tarceva), was approved by the Food and Drug Administration in 2004 for the treatment of locally advanced or metastatic non-small cell lung cancer (Pollack *et al.*, 1999). Also, ximelagatran (Exanta) was approved in Europe in 2004 as the first oral anticoagulant since warfarin to be marketed for almost 60 years, although it had to be withdrawn in 2006 due to toxicity issues post dosing. Ximelagatran is an example of a peptidomimetic derived from an understanding of how the key part of the peptide substrate of the protease binds to the enzyme active site (Gustafsson *et al.*, 2001).

Since 2005, there has been an acceleration of drugs reaching the market, derived using SBDD. For example, in 2006, five out of the total of 20 newly approved small molecule drugs were discovered using SBDD (Hegde and Schmidt, 2007). These were: dasatinib/Sprycel (BCR-ABL; oncology), sunitinib/Sutent (VEGFR/PDGFR/KIT; oncology), vorinostat/Zolinza [histone deacetylase (HDAC); oncology], darunavir/Prezista (HIV protease; HIV/AIDS) and sitagliptan/Januvia [dipeptidyl peptidase-4 (DPPIV); type 2 diabetes]. Interestingly, these drugs are not just directed at kinases, but a broader cross section of enzyme targets and include two entirely new drug targets, HDAC and DPPIV. More recently, there have been a number of other first-in-class agents launched where SBDD has been important. These include lapatinib, an ErbB2 inhibitor for cancer; aliskiren, a renin inhibitor for hypertension; and rivaroxaban, a Factor Xa inhibitor for venous thromboembolism. The development pipelines of many pharmaceutical

companies contain many further agents derived from SBDD approaches most notably the cathepsin K inhibitor odanacatib which is in development for osteoporosis and a number of hepatitis C virus (HCV) protease inhibitors for HCV infection (e.g. telaprevir and boceprevir). It is clear from the above that SBDD is now having a huge impact on the pipelines of pharmaceutical companies for targets which are amenable to protein crystallography. Several of the targets mentioned which have now yielded to small molecule drug development were at one time considered poorly druggable or undruggable and it is the structural insight that has enabled the discovery of clinical agents. The emerging field of protein-ligand X-ray crystallography for GPCR targets, where until very recently structural information was extremely limited, is likely to have similar benefits to medicinal chemistry against GPCR targets in the near future. In the remainder of this review, we will draw upon some examples to highlight the problems that have been solved using SBDD approaches and that might in future be applied to GPCRs.

GPCR small molecule binding sites

As described earlier, GPCRs are integral membrane proteins with seven transmembrane (TM) alpha helices. These helices surround a small molecule or peptide binding site within the TM domain. This overall architecture is highly conserved, despite the often limited sequence homology (Lagerström and Schiöth, 2008). The helices are linked by extracellular loops which vary significantly and in some cases play an important role in ligand recognition. There is much data to support the fact that small endogenous ligands bind within the TM binding site, based on mutagenesis data and from the recent protein-ligand X-ray structures of the β_1, β_2 and A2a GPCR proteins (Wess, 1993; Kristiansen, 2004; Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008). Although peptide ligands, such as peptide hormones, bind primarily to the extracellular portions of the receptor (including the N-terminus and the extracellular loops) in many cases, at least part of the peptides, either from the N or C-terminus, or a loop from the centre, binds within the TM binding site (Schwartz, 1994; Hibert *et al.*, 1999; Chen *et al.*, 2009). Upon binding of the endogenous agonist to the TM site, transduction of the signal through the membrane is driven by a conformational change to the receptor as a result of agonist binding. The mechanism of this is still not clearly understood, but it is believed that key contacts formed by the agonist within the ligand-binding site causes conformational changes through highly conserved conformational switches within the TM helices, leading to G-protein signalling (Kobilka, 2007; Nygaard *et al.*, 2009). This signalling mechanism dictates that a common TM trigger region must be present in all GPCRs and that it must be conformationally flexible so that it can move from an un-bound (or antagonist bound) conformation to an agonist-bound state for the signalling mechanisms to occur. This leads to the persuasive argument that a cavity suitable for binding of small molecules should be present in this region in all GPCRs; even for those whose ligands are peptides or proteins, and whether or not the natural ligand fully occupies the cavity.

These characteristics of the TM site make it an attractive target for drug discovery. In some cases, a small molecule agonist exists that can be the starting point for drug discovery efforts. Indeed, much of the profits of major pharmaceutical companies in the last 30 years have come from medicinal chemistry optimization of adrenaline (e.g. salmeterol) (Griffith, 2008) and 5-hydroxytryptamine (e.g. sumatriptan) (Glennon and Dukat-Glennon, 2008). Even in cases where the binding site interacts with a peptide ligand, the fact that the binding site is deep within the TM region means it is likely to have good overall features (active site topology) for interaction with small molecules. A recent example of this is the chemokine CCR5 receptor, for which the small molecule maraviroc was recently launched for the treatment of HIV infection. Five clinical candidates: maraviroc, vicriviroc, aplaviroc, TAK-779 and TAK-220 have been used to establish the nature of the binding pocket in CCR5 (Kondru *et al.*, 2008). Although the five antagonists are very different in structure, shape and electrostatic potential, mutagenesis data suggested that they were all able to fit in the same binding pocket formed by the TM domains of CCR5 rather than interfering with the protein–protein interaction site between the chemokine and the receptor (the orthosteric site). It is believed that the chemokine activates the receptor at least in part via its N-terminal residues within the edge of the TM site and maraviroc and other related CCR5 antagonists are allosteric ligands, preventing the switch to the agonist state. A similar situation is now thought to be the case for ligands of CXCR4, another HIV target and also a target in oncology. For example, the binding site for the launched drug Plerixafor has been shown to be within the transmembrane site (Wong *et al.*, 2008). These and other examples indicate that the TM binding site, conserved across the GPCR system (Barton *et al.*, 2006; Blaney *et al.*, 2006) to allow switching between antagonist and agonist states, forms a druggable site suitable for interaction with small molecules, even in the absence of an endogenous small molecule agonist. As further structures are solved, there is great potential for development of new drugs which are directed at this druggable site, rather than targeting peptide and protein–protein binding sites which are likely to be more difficult to target with small molecules (Wells and McClendon, 2007).

Allosteric regulators

The allosteric nature of the binding of miraviroc and other CCR5 antagonists is not unusual in the GPCR field. In fact, there are many examples of both negative allosteric modulators (NAMs) and positive allosteric modulators that bind outside of the (agonist) orthosteric site, but modulate the signalling of the receptor (May *et al.*, 2007; Milligan and Smith, 2007). A good example of this is the metabotropic glutamate receptor subfamily (mGluRs). There are eight subtypes within the mGluR group, which are part of the family C sub group of GPCRs. These receptors perform a variety of functions in the central and peripheral nervous systems and have been implicated in learning, memory, and the perception of pain (Simonyi *et al.*, 2005; Goudet *et al.*, 2009). Family C receptors have two distinctly separated topological

domains; a large extracellular amino-terminal domain (500–600 amino acids), which contains the agonist (glutamate) binding pocket and the 7TM domain involved in receptor activation and G-protein coupling (Pin *et al.*, 2004). Malherbe *et al.* (2003) have demonstrated, using an elegant mutagenesis study, that the NAM 1-ethyl-2-methyl-6-oxo-4-(1,2,4,5-tetrahydro-benzo[d]azepin-3-yl)-1,6-dihydro-pyrimidine-5-carbonitrile (EM-TBPC) is likely to bind within the TM binding site. A homology model (starting from rhodopsin) was constructed in which the binding site of the ligand was refined by measuring the extent of binding of radiolabelled ligand into receptors mutated in the TM domain. In particular, a number of mutations were identified that prevented ligand binding and clustered together inside the proposed TM binding cavity. The authors noted that these non-binding mutations were within the same region of the receptor as ligand recognition sites proposed for hβ2 adrenergic, hA_3 adenosine or h5-HT_4 receptors – that is, the allosteric site for mGluRs was co-located in a region analogous to the orthosteric agonist site for family A GPCRs. These and other data support the idea that allosteric modulation of GPCRs is often via interaction within the TM region, even when the agonist binds elsewhere to the receptor. As our structural understanding of these binding cavities improves, there will be much scope for application of SBDD approaches, such as virtual and fragment screening.

Virtual screening

Virtual screening has become a routine method in drug discovery in recent years. It is the process whereby the binding site of a protein target is interrogated using computational software with candidate small molecule ligands, each of which is 'docked' in one or more accessible conformations using a combination of shape matching and predictions of favourable hydrogen bonding and charge–charge interactions. The computational approaches used and examples of successful case histories have been reviewed elsewhere (Barril *et al.*, 2004; Ghosh *et al.*, 2006; McInnes, 2007). The overall aim is to identify a smaller number of compounds for screening than used in high throughput screening (HTS), but to achieve a much higher hit rate, improving the efficiency of the process. Virtual screening can also be used to identify compounds for purchase which are not present in the HTS collection, potentially identifying classes of hits that would otherwise not have been discovered. This 'enrichment' of hit rates by introducing a bias in the screening set can also help to improve the quality of the hits in terms of diversity and drug-like properties because the candidate molecules are often pre-filtered to remove lower quality compounds and compound classes which would not be considered novel by the chemists for the target being studied. Thus, the success of a virtual screen is best judged in terms of finding higher-quality scaffolds rather than a simple consideration of numbers of hits.

Integral to the method is access to high-quality structural information of the target binding site and preferably also information as to how one or more small molecule ligands bind in order to identify the 'hot spots' for binding. Typically, this will mean that there are one or more protein-ligand X-ray structures determined for the target. However, if the target itself does not have this information available, it may still be possible to derive a high-quality homology model of the binding site, suitable for virtual screening, if there is a highly homologous family member for which these data have been determined. For example, in the kinase field, there is often sufficient information to model targets with some confidence based on the available crystal structure information. In the GPCR field, the published crystal structures are now being used in combination with the rhodopsin structures to create homology models for close neighbours for use in virtual screening (Archer *et al.*, 2003; Deupi *et al.*, 2007; Engel *et al.*, 2008; Radestock *et al.*, 2008).

Fragment screening

A very popular approach to finding hits and leads for targets where structural information is available is to screen fragments. Fragments are very small molecules, usually defined as 100–250 Da in size or sometimes selected to obey the 'Rule of 3' whereby molecular weight must be less than 300 Da, lipophilicity (logP) must be less than 3 and the number of hydrogen bond donors or acceptors must be no higher than 3 (Congreve *et al.*, 2008; Chessari and Woodhead, 2009; Schulz and Hubbard, 2009). It is generally accepted that fragments must be screened at higher than normal concentrations to detect binding, given that due to their small size they have consequently lower affinity than drug-sized hits from HTS, and a range of biophysical approaches have been applied. These include 'SAR by NMR', ligand observed NMR methods such as water ligand-observed gradient spectroscopy or saturation transfer difference NMR, surface plasmon resonance, thermal denaturation and direct soaking of protein-ligand crystals for X-ray crystallography (Erlanson *et al.*, 2004). Although it is not an absolute requirement, structural information of how the fragment hits bind to the target protein is a powerful aid to their subsequent optimization. Fragment hits tend to have low affinity, albeit that their affinity per atom may in fact be higher than larger hit molecules (higher 'ligand efficiency') (Hopkins *et al.*, 2004). Fragment-based drug discovery has now established itself as a good way to identify ligands for poorly druggable targets. An example is β-site APP cleaving enzyme (BACE), a central nervous system (CNS) target for treatment of Alzheimer's disease, where a number of novel chemotypes have been discovered with the potential for developing CNS drugs for this very challenging target starting from fragments (Albert *et al.*, 2007; Keserü and Makara, 2009). Fragments are therefore good start-points for medicinal chemistry, but typically significant optimization is required to derive a drug molecule and this tends to be guided by the protein-ligand structure using SBDD methods.

Designing selective agents using protein-ligand structures

The close homology across the GPCR family and in particular between subtypes of receptors has been a major problem in

the design of safe and well-tolerated drugs for some receptor targets. A large number of GPCR drugs on the market are not particularly selective for the target of interest. Most notable are the antipsychotics such as clozapine and olanzepine. These have activity at as many as 12 different GPCR targets (Bymaster *et al.*, 1996). While some of these receptor activities contribute to the anti-psychotic activity, for example, D_2 and 5-HT_{2A} (Kuroki *et al.*, 2008), others mediate side effects such as weight gain (H_1) and cognitive deficits (M_1) (Robinson, 2007; Nasrallah, 2008). Improved selectivity between subtypes is also required to progress muscarinic agonists as cognitive enhancing agents, because activity at M_1 must be optimized relative to M_2 and M_3 which mediate cardiovascular side effects (Messer, 2002; Langmead *et al.*, 2008). Better selectivity is being sought in the development of 5-HT_{2c} agonists for the treatment of obesity. In this case, activity at 5-HT_{2b} must be avoided to reduce the potential for valvulopathy (Wacker and Miller, 2008).

Protein-ligand structures are a very powerful aid to interpreting and designing selectivity between close protein family members. One of the best examples of this is the area of oncology research and kinase inhibitors. There are more than 500 protein kinases in the human genome, involved in the complex web of cellular signalling throughout the body. Misregulation of signalling can be one of the drivers in oncology and inhibition of one or more kinases is now well validated as a targeted treatment of certain types of cancers. At the outset of research into kinase inhibition, there was large-scale scepticism that sufficiently selective compounds could be developed and a belief that new agents would be toxic as a result. However, as the field has developed, it has become clear that it is possible to design selective compounds, either to a single kinase or to a cross-section of kinases to give a particularly desirable inhibition profile (Ghose *et al.*, 2008).

Lapatinib (Tykerb) is a potent inhibitor of ErbB-2 (HER2/neu) and epidermal growth factor receptor (EGFR) kinase, approved for treatment of breast cancer in 2007. It is highly selective versus a broad range of other kinases (Wood *et al.*, 2004). The origin of this selectivity is revealed by its crystal structure in EGFR. The ligand binds to an inactive form of the kinase, in which the ATP site is relatively closed and a large pocket is created by a 9-Å shift in one end of the C-helix by a substituent on the molecule. The shift in position of the C-helix is significant because it results in the loss of a highly-conserved Glu–Lys salt bridge (Glu738 and Lys721), which is known to ligate the phosphate groups of ATP and is important in the mechanism of kinases. Lapatinib has a slow off-rate from the active site, possibly as a result of the requirement for a protein conformational change on its dissociation, and it is thought this influences the duration of drug activity *in vivo*. The use of structures to optimize ligand activity at different conformations should enable the rationale design of both agonists and antagonists.

A second strategy to be successfully applied in kinase research is to deliberately introduce a substituent onto the ligand in order to increase selectivity. Substituents are selected which should bind to the target protein but which are predicted to clash with residues in the active site of an anti-target. A recent example of this approach is given in the development of the AKT (PKB) inhibitor clinical candidate GSK690693 (Rouse *et al.*, 2009). The compound is a nanomolar inhibitor of AKT1, 2 and 3 but an aim of the project was to ablate binding to rho-associated, coiled-coil containg kinase 1 (ROCK1), inhibition of which is known to result in hypotension. Cross reactivity of AKT inhibitors to ROCK has been a serious issue in all compound series published and so not a trivial objective. Modelling of lead compounds in to AKT2 or ROCK1 revealed the potential to target a residue change in the pocket where a larger amino acid in ROCK (methionine) replaces leucine in AKT. Access to this region of the active site was through a narrow groove, and it required a number of iterations to identify a suitable narrow rigid substituent that gave over a 100-fold selectivity. Further optimization of pharmaceutical properties, while retaining this 'selectivity handle', gave the clinical candidate from the series. The origins of its selectivity were proven by solving the compound's crystal structure in AKT2, supporting the original docking studies.

A comparison of the recently published protein-ligand structures of the β1- and β2-adrenergic GPCRs shows great potential for the design of selective agents (Cherezov *et al.*, 2007; Warne *et al.*, 2008). Compared with close neighbours in kinases, the two receptors have more residue differences within and particularly on the periphery of the binding sites. As well as residue changes, there are also a number of rotamer changes to amino acid side chains. Very close to the ligands, there are two residue changes between β1 and β2; F325/Y308 and V172/T164. If we examine just one of these, the tyrosine (Y308) in β2 forms a hydrogen bond to asparagine 293, which is therefore absent in β1 where the phenyl alanine (F325) cannot do so. This has the effect of changing the positions of the aromatic rings of F325 and Y308 relative to each other and also of the nearby asparagine between the two systems, such that in β1 the asparagine is capable of forming a weak hydrogen bond to the ligand, while in β2 this residue cannot be involved in ligand binding in the same way (Figure 2). Overall, the top edge of the ligand-binding pocket made by these residue pairs has a subtly different shape and polarity, which would be expected to allow for the design of ligands with high selectivity. Unfortunately, the two solved ligands (cyanopindolol in β1 and carazolol in β2) are in fact non-selective as they do not form significant contacts with the regions that vary between the two systems. It would be fascinating to see additional structures solved with selective agents – efforts in our laboratories to this end are currently in progress.

Outlook

It is clear that the field of GPCR drug discovery has now reached a turning point in which rational drug design will start to replace empirical drug discovery. In particular, developing highly selective ligands and ligands with targeted polypharmacology (in an analogous way to kinase research) will start to become possible – something which has often been an insurmountable problem in the past, leading to many clinical failures due to side effects or lack of efficacy at safe doses. Equally important, targets which have to date been intractable should start to yield to small molecule drug

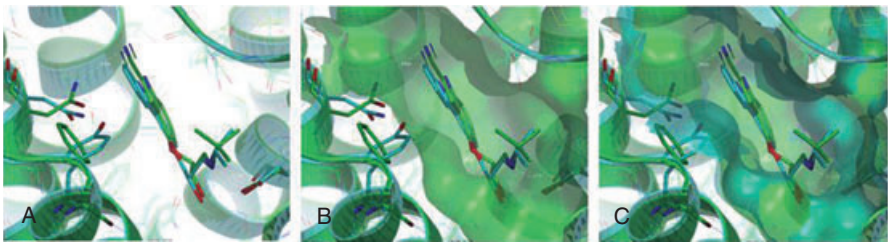

Figure 2 Superimposition of crystal structures of cyanopindolol in β1 and carazolol in β2 adrenoceptors (A) illustrating how the shape of the pocket in β1 shown in green (B) differs from the shape of the pocket in β2 shown in blue (C) [β1 green surface also shown in (C)]. These shape differences, coupled with specific amino acid residue changes described in the main text, suggest that more selective agonists and antagonists could be designed for these receptor targets.

discovery using structural insights, in an analogous way to the protease field where we have seen huge progress in recent years for targets which at one time were thought undruggable, such as Hepatitis C virus protease and BACE. Equivalent examples in the GPCR field include targets such as selective muscarinic M1 agonists and small molecule agonists for Family B peptide receptors. More fundamentally, we are now on the cusp of discovering at the molecular level the first step in how GPCR signalling actually works, namely, the structural changes that occur within the receptor during activation. The recent progress in obtaining GPCR structures will almost certainly open up further therapeutic opportunities for targeting GPCR pharmacology in the years to come.

Acknowledgements

Thanks to Nathan Robertson for preparing the figures. Thanks to Chris Langmead, Malcolm Weir and Frank Blaney for their critical reading of the manuscript.

Statement of conflict of interest

MC and FM are employed by Heptares Therapeutics, a company engaged in structure-based research for GPCR targets. Heptares Therapeutics is not involved with the development of any of the drugs referred to in this review.

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