Molecular Manipulation of G-Protein-coupled Receptors: A New Avenue into Drug Discovery

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Abstract: During the past 10 years or so, associated with the introduction of molecular biology techniques to G protein-coupled receptor (GPCR) research, outstanding progress has been made in understanding the mechanisms of action of these key proteins and their physiological functions. *in-vivo* manipulation of levels of GPCRs using transgenic and gene knock-out approaches have been particularly successful in assessing the roles of specific GPCRs in animal physiology.

Drug discovery is aiming to produce highly specific compounds based on subtle definition of receptor subtypes which can best be studied using heterologous expression of wild type or mutated forms of cDNA or genes encoding these proteins. Furthermore, new therapeutic opportunities may be provided by investigation of orphan receptors, the natural ligands for which remain unidentified. Some human diseases have been shown to be associated with rare mutations of GPCRs and the possibility that widely distributed polymorphisms in GPCR genes may allow selective therapeutic strategies for population subgroups is driving the development of the science of pharmacogenetics.

Introduction

Signal transduction in response to many hormones, neuropeptides, chemokines, odorants or chemical transducers for taste is initiated by their binding to members of a large family of heptahelical G protein-coupled receptors (GPCRs). Recent estimates suggest that approximately 1% of the proteins encoded by the human genome will belong to this family. These have been the most successful family of protein targets in small molecule drug discovery programmes with estimates that some 50% of the sales of clinically effective drugs are for agonist and antagonist ligands at these receptors.

GPCRs are located at the plasma membrane and represent the first element in signal transduction processes that drive many physiological functions. The first 2 GPCRs to be purified and cloned were bovine opsin [1] and the $_2$-adrenergic receptor [2]. From the sequence and likely structural similarities observed between these 2 proteins, despite wide differences in the ligands that activate them and in the signals they transduce, a large number of related proteins have very rapidly been identified with currently almost 2000 cloned GPCRs. They all comprise 7 membrane spanning domains linked by 3 extracellular and 3 intracellular loops, with an extracellular N-terminus and a cytoplasmic C-terminal tail.

With the application of molecular biology techniques such as cloning, DNA modifications (mutagenesis, deletions, chimaeric protein generation), expression in heterologous systems, and the production of animal models with modified gene expression, the face of pharmacology and small molecule drug discovery has changed radically. Novel GPCRs and splice variants of many GPCRs have rapidly been identified, allowing their incorporation into high throughput ligand discovery programmes without the absolute requirement for an easily observable physiological endpoint or bioassay. Indeed, in the case of "orphan" GPCRs for which the natural ligand has not been identified, selection for incorporation into ligand discovery programmes is often prioritised based on little more than their expression patterns as gleaned from *in situ* hybridisation studies. However, once either the natural ligand is identified or synthetic ligands discovered via high throughput screening, maps of key residues of the GPCR involved in ligand binding and the mode of coupling of the receptor to primary (G-proteins) or secondary effectors (secondary messenger regulators) can often be rapidly achieved [3], resulting in new insight into disease and futher refinement of the drug discovery process.

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After the first GPCRs were cloned, 3 major questions were addressed [4]:

1) what is the nature of the ligand binding site(s)?

2) which effector molecules are coupled to the GPCR?

3) what is the role of receptor heterogeneity ?

A large body of experimental data derived from a large number of cloned receptors, coupled with powerful computer modelling, has now produced a significant level of understanding of the first point. We also have substantial information on the mechanisms of coupling of GPCRs to G-proteins, but numerous unexpected effector outputs have been identified using transient or stable expression of GPCRs in different heterologous cell systems. The direct relevance of some of these reported signalling responses to the function of the GPCRs in physiological setting remains unclear. Furthermore, as many distinct GPCRs derived from different genes appear to function as the primary targets for a single neurotransmitter (e.g. 5 different receptor genes for dopamine and at least 13 for 5-hydroxytryptamine have been identified) then in the absence of highly selective ligands for closely related GPCRs unravelling the true physiological role for each can be a daunting process.

Although it was generally believed that GPCRs are inactive until they bind an appropriate agonist ligand, this idea is neither strictly feasible in thermodynamic terms or supported by experimental evidence often derived from high level heterologous expression of GPCRs. The existence of ligand-independent (also described as constitutive) activity has resulted both in a reassessment of mathematical models of GPCR function and alteration in basic pharmacological definition of drugs to include the term “inverse agonist” as a class of compounds which preferentially bind to and stabilise inactive conformations of GPCRs and thus reduce GPCR constitutive activity. By contrast, the previous general use of the term “antagonist” for an agent able to block the function of an agonist should now be restricted formally to ligands that bind with equal affinity to active and inactive conformation of a GPCR (or in a more prosaic manner, of two ligands concurrently applied, the one with the lowest efficacy will functionally “antagonise” the other, by competing for the GPCR binding site). As mutations in GPCRs which enhance constitutive activity have been observed in a growing list of relatively infrequent human diseases there is a clear basis for selective design of inverse agonists for effective treatment of these conditions. However, it remains a matter of contention whether inverse agonists are inherently likely to be more effective than true antagonists in most therapeutic settings [5], as the degree of constitutive activity noted in many physiological settings is not high.

The use of *in-vivo* animal models following genetic manipulation has allowed information to be gathered regarding the physiological role of GPCRs and the potential expression of further, previously unidentified but functionally closely related GPCRs, as in the example of the recent expansion in the family of protease-activated receptors (see [6] for review). Animal models for human diseases have been created using these techniques which consist of inserting into the animal genome a foreign DNA leading to overexpression of a given GPCR (transgene) or the deletion of this gene from the whole genome or in a tissue specific manner (knock-out). Antisense technology has also been used to eliminate gene products by injecting into host cells oligonucleotides designed to anneal to the pre-mRNA derived from a gene of interest. This approach has found particularly wide application for GPCRs expressed in the central nervous system where intra-cerebro-ventricular introduction of an antisense construct has resulted in substantial knockdown in levels of GPCR expression in a number of cases. All these techniques and their use in GPCR research were recently and comprehensively reviewed [7].

Defining the Ligand Binding Domains of GPCRs

A common structural basis for the GPCR family began to become apparent following the initial cloning studies. Bovine opsin and the $_2$-adrenergic receptor, displayed homologies to each other, particularly within the putative transmembrane spanning helices, and with a further, unrelated, protein, bacterio-rhodopsin. This led to the first steps in molecular modelling although bacterio-rhodopsin, despite sharing a number of structural features, particularly the 7-transmembrane elements, with GPCRs, did not couple to a G-protein. With the more recent 2-dimensional crystallisation of rhodopsin [8], molecular modelling of GPCRs and its use in drug discovery has been reinvigorated as this has resulted in the refinement of previous models based on the bacterio-rhodopsin structure. The first crystal of a GPCR is still lacking, although considerable effort has been directed to this end.

Structure-function analysis of cloned GPCRs allowed the identification of domains (use of chimaeric receptors) or residues (site directed mutagenesis) important for the binding of a specific ligand. Coupling analysis of the presumed binding pocket with a molecular model of the GPCR can at least result in post-hoc rationalisation of ligand docking and is likely to lead

to improvement in the design of new ligands, potentially with higher selectivity for closely related GPCR subtypes and certainly with higher binding affinity. Despite the lack of functional homology the use of bacterio-rhodopsin as a template for modelling of GPCRs has permitted an understanding of the binding of agonist and antagonist for a wide range of GPCRs (for review see [9-11], and [12] this issue).

For ligands which bind deep within the cleft formed by the molecular architecture of the 7-transmembrane helices such analyses are conceptually easier than for peptide ligands for which at least a number of the contact points are likely to be with amino acids on the extracellular face of the membrane. Despite this, considerable progress has been made on this basis for peptide ligand selectivity. Following are two examples of how the combination of cDNA modification and molecular modelling allowed a refined definition of the binding pocket for a peptide hormone receptor. Different approaches have been used to identify the binding site of both agonists and antagonists. One consists of a first pass identification of the portion of the GPCR important for high affinity binding by designing chimeras between 2 different GPCR which bind distinct but related ligands and then refining the study by site directed mutagenesis of the important residues arising from the previous step. Another approach attempts to identify the residues in the receptor that could possibly interact with the ligand, using molecular modelling and ligand docking. The model is then refined in an iterative manner based on the results of mutagenesis studies which are informed and selected based on the molecular model.

The first approach has been used to study non-peptide antagonist specificity between closely related GPCRs. The cloning of the cholecystokinin (CCK)-2 receptor (previously called the CCK-B receptor) first showed that the apparent difference between a CCK-2 and a gastrin receptor was due to the presence of species orthologues, the canine receptor differing from the human and the rat ones by only a few residues [13]. This variation resulted in a difference in the affinity for a non-peptide antagonist, and this was then used to determine which residues were important to confer high affinity to the antagonist at the human receptor. These authors identified potentially relevant specific residues on the basis of differences in ligand affinity measured in chimaeric receptors derived from the human and the canine GPCRs [14]. These specific residues were further tested by site-directed mutagenesis which showed that a single position was responsible for the different affinity observed for this compound. Ligand selectivity between CCK 1 and CCK 2 receptors could not be explained by this substitution and in a further study combining DNA modification and a rhodopsin-based model of the GPCR, it was shown that a binding pocket formed by a cluster of 8 important amino acids [15] was the main determinant of subtype specificity.

The second approach was chosen for identification of the binding site of the first non-peptide antagonist (BIBP 3226, R-N^2-(diphenylacetyl)-N-(4-hydroxy-phenyl)methyl-argininamide) at the neuropeptide Y (NPY) Y_1 receptor. A first model of possible interaction between this GPCR and its natural ligand was initially generated based on the bacterio-rhodopsin structure [16, 17]. Residues possibly involved in the binding of BIBP 3226 were then predicted on the basis of this model and tested for loss of function associated with appropriate single point mutations. The model was then refined accordingly, resulting in evidence for overlapping but distinct binding pockets for the agonist and the antagonist [18]. Another antagonist for this GPCR (SR 120819A) has also been designed using an approach based on the known interactions between NPY and the NPY Y_1 receptor [19].

Receptor Subtypes: Toward the Discovery of More Specific Ligands

As noted above, many endogenous ligands have the capacity to stimulate multiple GPCR subtypes. Certain receptor subtypes such as the histamine H_3 receptor possess little sequence similarity with other GPCRs which apparently utilise the same primary ligand [20]. Despite this, in general such GPCR subtypes are likely to possess significant sequence homology based on a shared requirement to bind the same ligand. However, it is common to observe that ligand binding to these subtypes result in activation of different types of G proteins and thence different signalling cascades.

This could depend on the tissues that express the subtypes but it is also well appreciated that pre- or post-synaptic receptors for the same ligand often produce opposite effects. Prior to the use of molecular biology in pharmacological investigations, GPCR subtypes had to be defined, largely, on the basis of agonist potency orders obtained from binding experiments in animal tissue membranes and effect on isolated organs. Co-expression of closely related subtypes can render it difficult to discriminate the physiological effect of an individual receptor in the absence of highly selective ligands. With the availability of the cloned versions of these different subtypes, as well as the possibility of their individual expression in heterologous cellular systems, it becomes more practical to study the function of each receptor subtype without interference from a highly related one binding the same ligand. However, such simple experimental models are unlikely

to provide explanations for well appreciated physiological phenomena such as synergy between receptor systems and the capacity of apparently sub-threshold levels of certain ligands to 'unmask' the presence of other GPCRs. A fascinating recent study has suggested that such phenomena may, at least in part, reflect the recruitment of intracellular GPCRs to the plasma membrane [21].

Evidence for subtypes for the $_2$-adrenoceptors originally derived from binding and functional studies [22]. Molecular cloning followed by expression in heterologous systems confirmed the presence of three distinct genes located on different chromosomes (in man on chromosomes 2, 4 and 10) [23]. However, generation of high affinity ligands which display strong selectivity between them has been difficult to achieve. As such, the generation of animals genetically lacking either one or combinations of the subtypes has proved to be the most effective means of attempting to correlate function with physiology [23].

In the case of receptors for dopamine only 2 subtypes were generally agreed using traditional ligand binding and functional data. Using homology cloning after the cloning of the $_2$-adrenoceptor cDNA, a first clone showing characteristics of the dopamine D2 receptor was isolated [24], followed by the cloning of the dopamine D1 receptor by 4 groups simultaneously [25-28]. Subsequently three other subtypes have been discovered, now called dopamine D3, D4 and D5, with the D5 being closely related to the D1 and the D3 and D4 receptors showing substantial similarity to the D2. Unlike the situation with the $_2$-adrenoceptor subtypes, the potential role of the D2-like receptors in a variety of neuropsychiatric and movement disorders has resulted in committed medicinal chemistry programmes producing ligands with substantial selectivity profiles for the D3 and D4 receptors which may result in potential therapeutic agents [29, 30].

The molecular basis for receptor subtype selectivity has also been explored for agonist ligands. Chimaeric receptors between $_1$ and $_3$-adrenoceptors, both involved in fat metabolism, has allowed an explanation for the subtype selectivity of phenethanolamine ligands based on differences between these 2 subtypes in the 7th transmembrane domain [31].

Identification of new receptor subtypes by homology cloning first raises the question of the function(s) of these receptors. By using *in situ* hybridization, possible functions outside the originally proposed physiological target could be identified. Furthermore, pharmacological tools may determine the presence of a given subtype by comparison of the agonist and antagonist profile in a given tissue or organ. This is the situation for the NPY Y5 receptor for which a putative effect on rabbit ileum contraction has been documented, outside of its acknowledged effect on feeding. This could reflect either a species heterogeneity or a new as yet unidentified subtype with a different tissue distribution [32].

GPCR Polymorphisms

A polymorphism is a variation in the sequence of a gene which occurs and is maintained with significant allelic frequency in a population. A considerable number of polymorphic variations are known to occur in the coding sequences of GPCR genes. The number of such polymorphic variations is likely to expand greatly in the near future in parallel with gene sequencing capacity. These will provide the necessary requirements to analyse equivalent gene sequences from significant numbers of individuals in attempts to understand whether variations in gene sequence could be considered as a risk factor in multifactorial diseases. Such application of "pharmacogenetics" to populations selected for clinical trials may improve the significance and thus success of many clinical trials. Many detectable polymorphic variants are unlikely to alter GPCR function, either because they are silent polymorphisms which would not be expected to alter the protein sequence coded for or because the alteration produced is conservative or located in a position of the GPCR not essential for ligand binding or signal transduction. However, clearcut examples of GPCR polymorphisms which substantially alter key features of protein function have been recorded. For example, a polymorphism has been discovered in the cytoplasmic tail of the human $_1$-adrenoceptor [33]. This rather common variation (26% of allele frequency) was shown in a heterologous expression system to provide constitutive activity to the receptor as well as increased agonist-induced signalling. Such examples indicate that genetic variations could be the basis for differences in individual pathophysiology and in therapeutic response to drug (−agonist or antagonist in this particular case). This will certainly be an area of considerable interest and potential in the near future. However, even for well appreciated GPCR polymorphisms there often remains considerable debate about their significance in disease susceptibility [34] and many of the studies conducted have either been contradictory or have had insufficient statistical power to produce clear-cut results. The $_3$ adrenoceptor is involved in regulation of fat storage and mobilisation [35]. A well-documented polymorphism in man has a tryptophan at position 64 instead of an arginine and is particularly prevalent in certain populations, such as the Pima indians, which

have a well appreciated propensity for weight gain and early onset of non-insulin dependent diabetes mellitus. When this variation was explored following heterologous expression the form with Arg^{64} displayed reduced basal and agonist stimulated adenylyl cyclase activity [36]. Although a range of reports indicate a relationship between this mutation and a tendency to obesity in humans [35] this conclusion is far from universal [34] and this provides a salutory lesson for those who may too easily attempt to utilise and extrapolate such data.

Although more difficult to analyse at this time, it may be that polymorphic variation in GPCRs genes, outwith the coding region, which alter the levels of expression of the protein either by modulating transcription or mRNA stability will in the end be substantially more important to overall variations in GPCR pharmacogenetics within populations than individual amino acid variations in the proteins themselves [37].

Constitutive Activity of GPCRs: Link to Diseases

It became obvious from the observation that peptides corresponding to short portions of the intracellular loops of GPCRs were able to bind and activate G proteins, that these segments of GPCRs were important for their coupling and that some allosteric transition, induced by ligand binding, was necessary to produce a conformation of the GPCR where these elements were able to activate G proteins. These findings led to the acceptance of the ternary complex model where a GPCR can exist in active or inactive conformations with agonists being able to stabilise the structure of the GPCR in an active conformation.

In reconstituted systems, the spontaneous activity of a receptor in absence of any ligand, is a well-established phenomenon [38,39]. The replacement of a short segment of the 3rd intracellular domain of the hamster $_{1B}$-adrenoceptor by the corresponding region of the $_{2}$-adrenoceptor leds to the unmasking of constitutively active mutants. Not only was the modified GPCR able to generate signal in the absence of ligand but also the affinity for agonist was increased substantially [40, 41]. The reciprocal mutations ($_{2}$-adrenoceptor with an element of the 3rd intracellular loop from the $_{1B}$-adrenoceptor) also produced a constitutively active $_{2}$-adrenoceptor [42]. In addition, this $_{2}$-adrenoceptor has been shown to be constitutively desensitised and phosphorylated [43]. A constitutively active mutant of the $_{2A}$-adrenoceptor was also obtained by single amino substitutions in the C-terminal part of its 3rd intracellular loop [44], leading to the conclusion that the native sequence in this region has been selected to constrain the GPCR in a largely inactive set of conformations in the absence of agonist. Recently, by using a charged hydrophilic sulfhydryl-specific agent (MTSEA) able to modify accessible cysteines in the binding pocket of the GPCR, it was shown that the constitutively active mutant of the $_{2}$-adrenoceptor alters the orientation of the 6th transmembrane domain [45], confirming that activation of a GPCR, either constitutively or in a ligand-induced manner, results in conformational changes. Similar conclusions as to reciprocal movements of transmembrane helices 3 and 6 during the activation process have also been derived from a range of approaches [46]. Recently, by combining site-directed mutagenesis and 3-D modelling, constitutive activity induced in the delta opioid receptor by mutation in the 3rd and 7th transmembrane domains indicated that these elements are involved in interhelical interactions which maintain the GCPR in its resting state [47].

A large body of evidence for the existence of constitutive activity in numerous GPCRs has accrued in recent years using both wild type and mutated GPCRs. For example the dopamine D5 receptor displays natural constitutive activity which is substantially greater than the closely related dopamine D1 receptor [48], as does the histamine H2 receptor when expressed in a heterologous system [49]. Mutational studies have indicated that many other elements of GPCR structure contribute to maintaining the inactive conformation. For example, mutations in the 5th and 6th transmembrane domains of the Luteinising Hormone (LH) receptor induce constitutive activity [50] as do alterations in the 3rd transmembrane domain of the $_{1B}$-adrenoceptor [51], particularly in the so called DRY domain which is very highly conserved in type I GPCRs. Mutations leading to constitutive activation of specific GPCRs have been documented in human diseases such as congenital night blindness (rhodopsin), Jansen-type metaphyseal chondrodysplasia (PTH related protein receptor), familial hypocalciuric hypercalcaemia and neonatal hyperparathyroidism (Calcium sensing receptor). Mutations have also been found in the Thyroid Stimulating Hormone (TSH) and LH receptors resulting in congenital hyperthyroidism or familial male precocious puberty [52]. In the TSH receptor, a 9 amino acid deletion in the 3rd intracellular loop, inducing constitutive activity in the receptor, appears to be one of the major causes of toxic thyroid adenomas [53].

More recently the existence of selective constitutive activity in regard of different effector end points was shown by the discovery of a mutation in the $_{2}$-adrenoceptor which induced constitutive activity of a Na^{+}/H^{+} exchanger without increasing basal adenylyl

cyclase activity [54]. Similar findings had previously been noted with equivalent mutation in the $_{1B}$-adrenoceptor [55].

Detailed study of the mechanisms underlying constitutive activity may allow a better understanding of the conformational alterations leading from stimulation of the receptor by a ligand to the activation of the effector(s) and it is now well accepted that receptors exist in an equilibrium between active and inactive states that can be displaced by agonists (towards the active states) or inverse agonists (towards the inactive states) [5].

The term “inverse agonist” derives from early data [56] showing that -carbolines were able to induce opposite effect to that of the agonist on the GABA$_A$ / benzodiazepine receptor complex and that this effect could be blocked by neutral antagonists. Since then, following the evidence of links between mutationally induced GPCR constitutive activity and disease, numerous compounds originally labelled as antagonists have been shown to act as inverse agonists (for review see [5, 57, 58]). The definition of compounds as inverse agonists rather than antagonists may be of importance. A transgenic mouse model with cardiac-specific overexpression of the $_2$-adrenoceptor displayed increased cardiac contractility in the absence of ligand that could be reversed by infusion of the $_2$-adrenoceptor inverse agonist ICI 118551 [59]. A corresponding transgenic animal generated using the constitutively active mutant of the $_2$-adrenoceptor did not show the same phenotype, due to low expression of the mutant GPCR. Receptor up-regulation, achieved by stabilising the GPCR protein by use of -adrenoceptor ligands [60,61] led to increased cardiac contractility [62]. This up-regulation of receptor number may well be a possible explanation for the observed tolerance development after prolonged clinical use in some patients (see [49, 57] for review).

Orphan Receptors, New Targets for Drug Discovery

In order to keep pace with the application of genomics in medicine and its impact on therapeutics, identification of novel targets for drug discovery represents one of the major research efforts in the pharmaceutical industry . About a quarter of sequences cloned for proteins belonging to the GPCR family have known endogenous ligands, leaving the vast majority of sequences with unknown ligands and unknown functions. As GPCRs are one of the major targets in drug discovery, identification of ligands and function for these “orphan” GPCRs is a high priority (for review see [63]). The recent discovery of the orexins by two different groups using either a ligand based- or a function based-approach, is a good example of how orphan GPCRs can find both a ligand and a function. Simultaneously, 2 groups identified the presence in the lateral hypothalamus of two peptides which they called hypocretins [64] or orexins [65]. The first group identified the peptides by screening mRNA selectively expressed in the lateral hypothalamus, a region known as a major regulatory centre for autonomic and endocrine homeostasis, whereas the second group discovered orexins as ligands for two closely related orphan GPCRs. This has resulted in rapid analysis of the effect of these peptides in feeding and energy metabolism in mice [66] and rats [67]. It has also been reported that the expression of the gene coding for the precursor of orexin (prepro-orexin) is down-regulated in obese mice [68]. Ligands have been found for other initially orphan GPCRs by mean of overlapping tissue distribution. For example, the autoradiographic distribution of an orphan GPCR mRNA [69] matched perfectly that of neuropeptide Y [70]. Many of these approaches require substantial investment of time and energy in purification of peptide ligands or a degree of serendipity to produce significant progress. As such, the development of sensitive novel assay screens which can allow orphan GPCRs to be rapidly incorporated into high throughput screening programmes and hits from these to be optimised in medicinal chemistry programmes remain major priorities. Generally it may be easier to evaluate the likely function of an orphan GPCR in behavioural studies once a candidate antagonist has been identified than by attempting to match distribution patterns of novel peptides with orphan GPCRs.

Conclusion

The introduction, less than 20 years ago, of molecular biology to GPCR research introduced not only a greater knowledge of the basic mechanisms of known GPCRs functions, but also modified thinking on the design of new compounds that bind to them. Powerful computer software has allowed construction of probable images of these receptors, despite the lack of true structural knowledge, by calculating interactions between ligand and receptor derived from chemical structure-activity series and information arising from mutational studies. Design of peptide receptor ligands, for example, has greatly benefited from this combination of approaches [71].

The coupling of GPCRs to effectors has been studied intensely but the lack of detailed information on the structures of intracellular loops of GPCRs still ensures that it is difficult to accurately predict *a priori* the likely G protein selectivity of a newly identified and

structurally distinct GPCR. The application of molecular biology has, however, resulted in the identification of a plethora of new GPCRs as potential therapeutic targets and the capacity to express these in heterologous systems allows their rapid incorporation into ligand discovery programmes.

Abbreviations

GPCRs = G protein-coupled receptors

LH = Luteinising Hormone

TSH = Thyroid Stimulating Hormone

NPY = neuropeptide Y

CCK = Cholecystokinin.

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