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► **To cite this version:**

Nicola J. Smith, Kirstie A. Bennett, Graeme Milligan. When simple agonism is not enough: emerging modalities of GPCR ligands. *Molecular and Cellular Endocrinology*, 2010, 331 (2), pp.241. 10.1016/j.mce.2010.07.009 . hal-00654484

HAL Id: hal-00654484

<https://hal.science/hal-00654484>

Submitted on 22 Dec 2011

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Accepted Manuscript

Title: When simple agonism is not enough: emerging modalities of GPCR ligands

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PII: S0303-7207(10)00370-9
DOI: doi:10.1016/j.mce.2010.07.009
Reference: MCE 7596

To appear in: *Molecular and Cellular Endocrinology*

Received date: 15-1-2010
Revised date: 15-6-2010
Accepted date: 13-7-2010

Please cite this article as: Smith, N.J., Bennett, K.A., Milligan, G., When simple agonism is not enough: emerging modalities of GPCR ligands, *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.07.009

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When simple agonism is not enough: emerging modalities of GPCR ligands

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Abstract

Recent advances in G protein-coupled receptors have challenged traditional definitions of agonism, antagonism, affinity and efficacy. The discovery of agonist functional selectivity and receptor allostereism has meant researchers have an expanded canvas for designing and discovering novel drugs. Here we describe modes of agonism emerging from the discovery of functional selectivity and allostereism. We discuss the concept of ago-allostereism, where ligands can initiate signaling by themselves and influence the actions of another ligand at the same receptor. We introduce the concept of dualsteric ligands that consist of distinct elements which bind to each of the orthosteric and an allosteric domain on a single receptor to enhance subtype selectivity. Finally, the concept that efficacy should be defined by the activity of an endogenous ligand will be challenged by the discovery that some ligands act as 'super-agonists' in specific pathways or at certain receptor mutations.

Keywords

G protein-coupled receptor, Functional selectivity, Allostereism, Ago-allostereism, Dualsteric ligand, Super-agonism.

For decades now, the seven transmembrane-spanning (7TM) family of G protein-coupled receptors (GPCRs) has been a fruitful source of drug targets for the pharmaceutical industry, with blockbuster drugs acting at numerous GPCRs, most notably the monoaminergic (such as histamine, serotonin and the catecholamines) and angiotensin receptor families, constituting over 26% of all FDA-approved drugs (Overington et al., 2006). However, few recently approved GPCR-directed medicines regulate previously untapped pharmacological targets, in spite of concerted drug discovery programs within the pharmaceutical industry.

Failure to substantially expand the number of truly 'druggable' GPCR targets may be due to a number of reasons. Until very recently, little structural information has existed for GPCRs, meaning that rational drug design has had to rely upon homology modeling using the low homology template of rhodopsin (Michino et al., 2009, Mobarec et al., 2009). The recent publication of three high resolution crystal structures of clinically important GPCRs has therefore significantly expanded our understanding of how a ligand may gain access to and bind within the receptor binding pocket (Cherezov et al., 2007, Jaakola et al., 2008, Kobilka and Schertler, 2008, Rasmussen et al., 2007, Rosenbaum et al., 2007, Rosenbaum et al., 2009).

Most successful GPCR drugs on the market have been developed from the knowledge of and structure-activity relationships around an existing ligand template, such as adrenaline (Griffith, 2008). However, roughly 100 non-

chemosensory GPCRs remain to be 'deorphanized', in that they are yet to be matched to an endogenous ligand partner (Chung et al., 2008). Of those recently deorphanized, many of the identified ligands have poor potency and undefined affinity at the receptor (for example, the free fatty acid receptor family (Stoddart et al., 2008), GPR35 (Wang et al., 2006a), GPR109A and GPR109B (Tunaru et al., 2003, Wise et al., 2003), GPR120 (Hirasawa et al., 2005) and GPR84 (Wang et al., 2006b)) and are therefore a challenge for rational drug design.

Probably the biggest contributor to the lack of new ligand classes is the way in which chemical libraries have traditionally been screened (Kenakin, 2009b, Langmead and Christopoulos, 2006); high throughput screening of libraries has often been limited to a single readout, be it receptor binding, G protein activation or second messenger generation. In some cases, targets are even modified such that receptor signaling is directed towards a non-native readout, for example via the co-expression of chimeric or promiscuous G proteins (Milligan and Kostenis, 2006). Such a streamlined approach to drug discovery has undoubtedly advanced GPCR research efforts but two key ligand actions were overlooked by such approaches, namely allosterism and functional selectivity. In the present review, we will briefly introduce the concepts of allosterism and functional selectivity and highlight the exciting prospects for novel agonist medicines to emerge from a more detailed understanding of these phenomena. An appreciation of the newer modalities of agonism should contribute to the

discovery of more potent and selective agonists at hitherto untapped drug targets.

Functional Selectivity and Allostereism at GPCRs

Functional Selectivity

The notion of functional selectivity [also known as or encompassed by the terms biased agonism, agonist-directed trafficking of receptor stimulus, pleiotropy, or pluridimensional efficacy (Galandrin et al., 2007, Kenakin, 2007, Kenakin, 2008, Urban et al., 2007)] has arisen from the accumulation of experimental evidence showing that certain ligands, or certain biological systems, favor activation of one signaling pathway over another and is most likely a common phenomenon across many receptor types. As such, while an endogenous full agonist is theoretically capable of activating all of the possible pathways that its cognate receptor can couple to (provided it is in the appropriate cellular background), another ligand may selectively activate only a subset of endpoints, presumably because only a subset of receptor conformations are stabilized by the interaction. Functional selectivity is commonly determined through comparison of the activities of two or more ligands across multiple signaling pathways, thus functional selectivity is usually manifested by changes in rank order of efficacy, i.e. the extent of responsiveness of the system, and/or by changes in rank order of potency of different ligands acting at the same receptor. Changes in efficacy have been demonstrated, for example, at the pituitary adenylate cyclase-activating peptide (PACAP) receptor, where the agonists PACAP₁₋₂₇ and

PACAP₁₋₃₈ stimulate PACAP-receptor mediated adenylate cyclase activation with equal potencies, but only PACAP₁₋₃₈ could evoke an increase in inositol phosphate levels through activation of phospholipase C β (Spengler et al., 1993). For the Gonadotropin Releasing Hormone (GnRH) receptor, the two endogenous agonists, GnRH I and GnRH II, display functional selectivity as measured by differences in rank order of potency at more distal endpoints (Millar et al., 2008) – GnRH I is more potent than GnRH II at stimulating gonadotrophin release, while the rank order of potency is reversed for inhibition of cell growth (Maudsley et al., 2004). Both changes in the rank order of potency and efficacy of compounds have been observed at the human D_{2L} receptor expressed in CHO cells. For example, the compound S(+)-propylnorapomorphine (SNPA) acts as a full agonist with efficacy equal to quinpirole in inhibiting forskolin-stimulated cAMP accumulation, but acts with lower efficacy and potency than quinpirole in activating MAP kinase phosphorylation (Gay et al., 2004).

While alterations in potency can sometimes be explained by receptor reserve, the general phenomenon of functional selectivity is distinct to and not an artifact of receptor reserve (receptor reserve is unique to each agonist in the system). For example, for the dopamine receptor it is known that there is a higher pre-synaptic D₂ receptor reserve than post-synaptic receptor reserve (in a situation where there is high receptor reserve a partial agonist may have increased potency and efficacy), however, the agonist dihydrexidine has greater functional activity at post-synaptic D₂ receptors than pre-synaptic (Mailman, 2007). Instead,

functional selectivity is thought to derive from ligands stabilizing GPCRs in specific conformations that favor activation of a particular downstream effector. A number of elegant examples of distinct receptor conformations stabilized by ligands with different efficacies have been provided for the beta2-adrenoceptor; for example, Kobilka and colleagues (Ghanouni et al., 2001a, Ghanouni et al., 2001b, Swaminath et al., 2004) have used an environmentally-sensitive fluorophore to demonstrate two distinct fluorescence lifetimes upon full agonist stimulation with isoproterenol, while the partial agonist dobutamine only stabilized one of the observed conformations. These conformations were later found to correspond to specific ligand-mediated outcomes (Swaminath et al., 2005). More recently, multiple conformations of the purified and reconstituted beta2-adrenoceptor have been identified based upon their propensity to couple to purified $G\alpha_s$ in the absence or presence of guanine nucleotides (Yao et al., 2009). Meanwhile, in a very recent study, Bokoch and colleagues (2010) used solid-state NMR to identify changes within the extracellular surface of the receptor in response to ligand binding. Interestingly, they found that ligands with different efficacies that bound within the transmembrane section of the receptor were able to induce conformational changes in the extracellular space (monitored at a salt bridge formed between extracellular loops 2 and 3) to different degrees (Bokoch et al., 2010). In support of the beta2-adrenoceptor studies, molecular modeling in combination with site-directed mutagenesis has been used for the GnRH receptor to support the hypothesis that GnRH I and GnRH II stabilize distinct conformations of the receptor by making a series of different contacts

within the binding pocket in addition to their shared binding interactions (Millar et al., 2008).

Allosteric modulators

Conventional agonists target the same binding site on a receptor as the endogenous ligand, termed the orthosteric site; however ligands may also bind to a topographically distinct site on the receptor, referred to as an allosteric site. Binding of an allosteric ligand to a receptor can modulate the potency/affinity and/or the efficacy of the orthosteric ligand by altering the global conformation of the receptor compared to binding of the orthosteric ligand alone. Although by definition the binding site of an allosteric modulator is distinct from the orthosteric site (Neubig et al., 2003), in general, binding sites for allosteric modulators are not well defined but can be located close to (Christopoulos and Kenakin, 2002) (e.g. the muscarinic acetylcholine receptor family (Birdsall and Lazareno, 2005); and the free fatty acid 2 receptor (Lee et al., 2008, Milligan et al., 2009)) or far from the orthosteric site (e.g. the metabolic glutamate receptors (Knoflach et al., 2001)). Although this review is focused on ligands as allosteric modulators, the most common allosteric modulators of G protein-coupled receptors are the G proteins themselves (Milligan, 2005); the role of these and other interacting proteins, such as receptor activity modifying proteins, have been reviewed extensively elsewhere (Kenakin, 2008, Kenakin, 2009a).

Allosteric modulators are well-established therapeutic agents of ion channels but until recently have not been a traditional focus of drug discovery efforts at GPCRs (Conn et al., 2009). However in the last few years two allosteric modulators have entered the market as therapeutic targets of GPCRs: cinacalcet, a modulator of the calcium-sensing receptor (Harrington and Fotsch, 2007), and maraviroc, a modulator of the chemokine receptor CCR5 (Dorr et al., 2005), with the allosteric modulator LY2033298 acting at the muscarinic M₄ receptor showing potential as an anti-psychotic agent (Chan et al., 2008).

Emerging Agonistic Modalities

Allosteric agonists and ago-allosteric modulation

As introduced above, allosteric modulators can act to modify an orthosteric ligand's affinity for a receptor and/or modulate its efficacy, events which are both saturable and probe-dependent [i.e. depend upon the ligands being studied (Leach et al., 2007, May et al., 2007b)]. Additionally, a ligand binding at an allosteric site can have efficacy in its own right; this is termed an allosteric agonist or ago-allosteric modulator and is accounted for by the Allosteric Two-State Model (Langmead and Christopoulos, 2006, May et al., 2007b, Schwartz and Holst, 2006).

Ago-allosteric modulation is not a new phenomenon; it was first described in 1990 at the adenosine A₁ receptor where the allosteric modulator PD81723 was shown to not only activate the G $\alpha_{i/o}$ pathway on its own but also to enhance the

binding of the orthosteric radioligand [^3H]-cyclohexyladenosine when both ligands were present (Bruns and Fergus, 1990). Indeed, ago-allosteric modulation has also been described at a wide variety of receptors including the GABA_B receptor (Binet et al., 2004), the M₁ and M₂ muscarinic acetylcholine receptors (Langmead et al., 2006, May et al., 2007a), the free fatty acid 2 receptor (Lee et al., 2008, Milligan et al., 2009), and the glucagon-like peptide 1 receptor (Teng et al., 2007). Synthetic ago-allosteric modulators were also described for the ghrelin receptor (Holst et al., 2005) although further studies have suggested that these compounds act as simple orthosteric agonists (see section below on super-agonists).

In addition to their modulatory and agonistic qualities, some ago-allosteric modulators have also been suggested to display functional selectivity. For example, Thomas et al. (2008) demonstrated differential G protein coupling depending upon whether the ligand was an allosteric modulator or allosteric agonist at the M₁ mACh receptor (Thomas et al., 2008). More recently, Thomas et al. (2009) extended these studies to demonstrate that the ago-allosteric modulator, AC42, did not initiate receptor internalization and down-regulation, unlike traditional orthosteric ligands (Thomas et al., 2009). For the M₄ mACh receptor, the ago-allosteric modulator LY2033298 was found to modulate signaling via [^{35}S]GTP γ S, extracellular signal-regulated kinases 1 and 2 or glycogen synthase kinase 3 β to different degrees (Leach et al., 2010). In an elegant follow up to this study, Nawaratne et al. (2010) have used site-directed

mutagenesis to identify key contacts or domains within the M₄ mACh receptor for the ago-allosteric properties of LY2033298. Intriguingly, they were able to dissect out individual regions responsible for each of allosteric binding, allosteric agonism and the communication between orthosteric and allosteric sites resulting in modulation of either binding or function (Nawaratne et al., 2010). Clearly, ago-allosteric modulators may prove to be useful tools for regulating distinct receptor signals.

Allosterism across a hetero-dimer

Given the evidence that GPCRs can form dimers, or higher oligomer structures, it is not inconceivable that allosteric modulation may occur across the dimer interface (Milligan and Smith, 2007). Indeed, evidence to support allosteric modulation in receptor dimers has been supplied by studies into allosteric regulation of the γ -aminobutyric acid (GABA)_B receptor. The GABA_B receptor is an obligatory hetero-dimeric receptor composed of two subunits; GABA_{B1} and GABA_{B2} (Jones et al., 1998, Kaupmann et al., 1998, White et al., 1998). Interestingly, Binet and colleagues demonstrated that the positive allosteric modulator CGP7930 activated a mutant receptor that consisted solely of the GABA_{B2} subunit. As the endogenous ligand GABA binds solely to the GABA_{B1} subunit, this data suggests CGP7930 could be imparting its allosteric effect across the dimer (Binet et al., 2004).

The emergence of ago-allosterism has a number of implications for drug discovery. Firstly, allosteric binding sites are considered to have been under less evolutionary pressure for their maintenance than the orthosteric binding site of a GPCR and, therefore, are likely to display heterogeneity across a receptor family. Thus, ago-allosterism provides an opportunity for more selective agonism. Furthermore, allosteric modulators have been heralded as a safer alternative to orthosteric ligands as they exhibit a maximal 'ceiling effect', that is, their effect on affinity or efficacy of an orthosteric ligand is saturable. However, a number of ago-allosteric modulators appear to be intrinsic full agonists with efficacy equivalent to that of the endogenous ligand, despite binding to a distinct site – this would appear to negate the clear benefit of positive allosteric ligands in overdose, and also makes determination of experimental parameters of allosterism difficult. In light of increased appreciation of functional selectivity, it is becoming more important to measure many functional endpoints. This becomes an onerous task once the additional complication of probe-dependence of allosterism is considered – which ligands should be used as orthosteric probes to assess the allosteric effects of an ago-allosteric modulator? To extend the problem further, which animal models would be suitable to measure physiological and pathological endpoints of a pre-clinical ago-allosteric modulator? There are many examples in which orthosteric ligands identified initially at a human GPCR expressed in a heterologous cell line display significantly different potency and/or efficacy at species orthologues of the GPCR. This poses major challenges for translation of basic pharmacological information into animal models, and if

allosteric sites are indeed less well conserved throughout mammalian species than orthosteric binding sites, these issues of translation will undoubtedly generate even more substantial challenges.

Efficacy and super agonism

The term efficacy was coined by Stephenson (Stephenson, 1956) and refers to the ability of a ligand, once bound to a receptor, to elicit a physiological or pharmacological response. The parameter most frequently used to assess efficacy is the maximal agonist effect (E_{\max}), which is the maximal asymptote of a concentration-response curve fitted to experimental data (Strange, 2008). Efficacy is a relative phenomenon and so is usually assessed by comparing the E_{\max} of the test ligand to that of another ligand, most commonly the endogenous agonist (although this is clearly not practical for an orphan receptor). This allows ligands to be characterized either as full agonists (ligands that produce the 'maximal' response i.e. the maximum possible response produced by a ligand in the particular cell/tissue of study), partial agonists (ligands that produce a sub-maximal response) or antagonists (ligands that bind to receptor but do not possess efficacy). The relative nature of efficacy, however, means that each time a new ligand is identified as having the maximal response in a system, ligands that were previously considered to be full agonists are subsequently re-classified as partial agonists. Whilst pharmacologically correct, such an approach can be cumbersome and draw focus away from the endogenous agonist. This has led to some commentators to coin the term 'super agonism' to describe situations

where synthetic orthosteric ligands that share an overlapping binding site with the endogenous ligand(s) have been demonstrated to activate the receptor with higher efficacy than the endogenous agonist (Niemczyk et al., 2010, Tan et al., 2002).

Super agonists may prove to be useful therapeutic ligands. The non-peptide growth hormone secretagogue MK-677 is currently undergoing clinical trials as its growth hormone-releasing properties may be clinically useful for the treatment of idiopathic short stature or ageing (in which there is a natural decline in GH release). In one short (2 year) clinical trial healthy older adults who received MK-677 showed sustained increases in amplitude of pulsatile growth hormone secretion to levels observed in young adults (Nass et al., 2008). Interestingly, MK-677, (along with two other synthetic agonists, growth hormone releasing peptide-6 and L-692,585) has been described as a super agonist of the ghrelin receptor in the $G_{\alpha 01}$ pathway (Bennett et al., 2009). In a separate study, MK-677 was also described as a super agonist of the ghrelin receptor in β -arrestin mobilization studies and in serum-responsive element mediated transcription assays (Holst et al., 2006). Although the benefits of MK-677 in the treatment of growth hormone disorders has not been linked to its enhanced efficacy per se, MK-677 has been shown to act as a super agonist and may well become a clinically useful drug.

The study into activation of the ghrelin receptor by Holst et al. (2005) also demonstrates an important point - that efficacy varies depending on the assay

used to measure it, with MK-677 shown to act with efficacy equal to that of the endogenous ligand, ghrelin, in calcium mobilization and inositol phosphate accumulation assays (Holst et al., 2005). It has long been known that efficacy of a ligand may vary depending on the assay used. Thus, measuring efficacy in downstream assays where a degree of response amplification occurs can result in a masking of differences in ligand efficacies. Measurement of efficacy in downstream systems also means that feedback, cross regulation and even convergence of different effector systems may also be integrated into the measured signal, complicating experimental results (Wess, 1998). Thus it is best to measure maximum efficacy in response systems close to the receptor where few compounds will reach the maximal system response (Strange, 2008). Such an approach may reveal hitherto unrecognized super agonists and may highlight functional selectivity. It could be argued that super agonism is purely an artifact of, for example, alterations in receptor desensitization/recycling rates or alterations in ligand affinity. However, a study by Engel et al. (2006) demonstrated, for the thyrotropin-releasing hormone 1 receptor, that the thyrotropin-releasing hormone (TRH) analogue R-desaza TRH ((1R)-(3-oxocyclopentyl-His-ProNH₂)) exhibited a lower potency but a higher efficacy than TRH at similar occupancy of the receptor. By using ligands that affect receptor recycling or inhibit specific protein kinases, such as the selective protein kinase C inhibitor, Ro-31-8425, Engel et al. suggested that R-desaza TRH still acted as a super agonist with respect to TRH signaling (Engel et al., 2006). Although the effects of various G protein-coupled receptor kinases (GRKs) or regulators of G

protein signaling (RGS) proteins should be investigated before firm conclusions are drawn, it is possible that super agonism may not be an artifact of altered receptor number at the cell surface but is indeed a result of altered receptor-G protein interactions.

Dualsteric ligand design

The concept of a ligand binding simultaneously to more than one site on a GPCR and resulting in enhanced binding, efficacy or duration of signal is not new; for example, the beta2-adrenoceptor agonist, salmeterol, is thought to be long-acting because the extended aliphatic chain is tethered to a site on the beta2-adrenoceptor that is distinct from the orthosteric site (although there is no suggestion that this interaction is allosteric;(Griffith, 2008)). However, the rational design of orthosteric agonists that possess enhanced receptor subtype selectivity due to additional binding at an allosteric site is a new and exciting approach to drug discovery. For the purpose of this review, we will define such chemicals as dualsteric ligands (rather than the terms bitopic or hybrid ligands, that are also in current usage) to reflect the presence of both orthosteric and allosteric moieties and to distinguish them from heterobivalent ligands spanning across a homo- or hetero-dimer (e.g. KDN-21, which spans delta and kappa opioid receptors, (Bhushan et al., 2004)) or binding to two separate GPCRs (e.g. a peptide co-agonist at glucagon and glucagon-like peptide 1 receptors, (Day et al., 2009)).

It is no coincidence that the majority of studies to date examining dualsteric ligands have focused on the muscarinic family of acetylcholine receptors (mAChRs). Consisting of five subtypes, M₁-M₅, the highly conserved nature of the orthosteric binding pocket has meant that few selective agonists have progressed to clinical trials, despite a high degree of confidence that these would be appropriate therapeutic targets (Hulme et al., 1990). In the case of M₁ agonists for the treatment of the early stages of Alzheimer's disease, at least five compounds have failed to progress from early trials to the clinic owing to poor selectivity resulting in cardiovascular side effects via M₂ and M₃ mAChRs (Langmead et al., 2008). While the orthosteric binding pockets of the mAChRs are poorly conserved, considerable divergence is found in the allosteric sites of this family of GPCRs (Christopoulos and Kenakin, 2002). Thus, the combination of potent orthosteric moieties with near-neutral allosteric compounds into a single hybrid ligand would provide an ideal mechanism for conferring increased subtype selectivity. Indeed, close examination of one existing subtype-selective ligand, McN-A-343, has revealed its mode of binding to the M₂ mACh receptor to be bitopic (Valant et al., 2008, Valant et al., 2009) and it is conceivable that other ligands with clear selectivity interact with their preferred GPCR in the same manner. Holzgrabe and colleagues (Disingrini et al., 2006) employed a rational approach to the design of bitopic ligands and synthesized a panel of hybrids incorporating the potent yet non-selective agonists, oxotremorine, oxotremorine-M and analogues, and the M₂ mAChR-selective negative allosteric modulators W84 or naphmethonium. Although an agonist backbone was used to target the

orthosteric binding site, the dualsteric ligands generally displayed antagonism in a range of organ preparations and intrinsic efficacy was only revealed in a [³⁵S]-GTPγS measure of G protein activation in membranes from a heterologous cell culture system. Little subtype selectivity was observed. In contrast, reasonable subtype selectivity for the M₂ mAChR was obtained by Steinfeld et al. (2007), who generated a high affinity antagonist, THRX-160209, comprising a benzhydryl group linked to a 4-aminopiperidine motif by a C7 polymethylene chain (Steinfeld et al., 2007).

A very recent example of the promise of dualsteric ligands comes from Mohr and colleagues (2009), who extended the approach of Disingrini et al. above (2006) by again using high affinity M₂ mAChR-selective allosteric fragments but this time fused to the potent orthosteric muscarinic agonist, iperoxo (Antony et al., 2009). The authors successfully created dualsteric ligands that were able to act as full agonists with good selectivity for the M₂ mAChR both *in vitro* and in whole tissue pharmacology experiments. Unlike THRX-160209, which displayed markedly enhanced affinity for the receptor compared to its individual components (Steinfeld et al., 2007), neither hybrid 1 nor hybrid 2 displayed affinity or potency greater than iperoxo, the orthosteric building block (Antony et al., 2009). Thus, despite the expectation that fusing two separate binding partners into a single molecule would be energetically favorable and confer enhanced affinity upon the resultant dualsteric compound, it is clear that the nature of the ligand fragment influences the measured receptor binding or function (May et al., 2007b).

Not only can subtype selectivity be enhanced by incorporating allosteric and orthosteric moieties into a dualsteric compound, but functional selectivity can be achieved, highlighting the promise for this approach in disease states where activation of only a subset of pathways from a receptor is desirable (e.g., beta-arrestin2-mediated inotropy and lusitropy (Rajagopal et al., 2006) without G protein-mediated cardiac hypertrophy for angiotensin receptors [reviewed in (Smith and Luttrell, 2006)] or β -arrestin-dependent cardioprotection (Noma et al., 2007) without cardiac hypertrophy or progression to heart failure (Patel et al., 2009)). When investigating the nature of agonism to result from the dualsteric ligands hybrid 1 and hybrid 2, whole cell biophysical experiments in the presence of the $G\alpha_{i/o}$ inhibitor, pertussis toxin, demonstrated that while the endogenous agonist, acetylcholine, and the original orthosteric template, iperoxo, were still able to cause a cellular response, the dualsteric agonists were rendered inactive (Antony et al., 2009, Kebig et al., 2009). Thus, the dualsteric ligands were functionally selective for the $G\alpha_{i/o}$ pathway.

So what distinguishes dualsteric ligands from ago-allosteric modulators? Ago-allosteric ligands both bind and activate the receptor by interacting with the allosteric binding site, meaning that an orthosteric ligand is able to co-bind to the receptor and that any observed agonism would be the resulting combination of orthosteric agonism, allosteric-site agonism and any co-operativity that occurred between the two sites. In contrast, dualsteric agonists bind simultaneously to the

orthosteric and allosteric sites and activate the receptor via the orthosteric site: this was demonstrated by competition experiments in [³⁵S]GTPγS assays, where the interaction between the allosteric modulator and M₂ mAChR hybrid 1 ligand was found to be competitive (indicating that the dualsteric ligand bound at the allosteric site of the receptor) as was, at lower concentrations of antagonist, the interaction between hybrid 1 and the orthosteric antagonist atropine (Antony et al., 2009). Interestingly, at higher concentrations of atropine, hybrid 1 was no longer able to competitively overcome atropine binding and the rightward shift appeared to be saturating (negative allosteric modulation) – the authors concluded that this reflected the displacement of the agonistic fragment of hybrid 1 from the orthosteric binding site by atropine (hence the reduction in signal) but the retention of allosteric fragment binding at the allosteric site (Antony et al., 2009). Thus, although dualsteric ligands appear to exert their agonistic effects via the orthosteric site, their pharmacology has the potential to be equally if not more complicated than an ago-allosteric modulator because the ligand can act as an agonist at the orthosteric site, an allosteric modulator if the orthosteric site is bound by another ligand and an antagonist for either allosteric or orthosteric ligands. The capacity for the allosteric moiety of a dualsteric ligand to bind to the receptor without the agonistic fragment occupying the receptor means that if the rationale for designing a dualsteric ligand was to obtain receptor subtype selectivity, the allosteric fragment should ideally have little to no co-operativity. Otherwise, orthosteric agonist efficacy may be affected (May et al., 2007b).

Concluding remarks

Understanding of the mode of action of synthetic ligands that target members of the G protein-coupled family of receptors has recently been extended greatly by detailed analysis, often in parallel, of multiple elements of their ability to bind and/or activate receptors. Some of the emerging modalities offer distinctly novel avenues to drug discovery that are likely to revitalize programs of work on receptors that had appeared intractable or where receptor subtype side effects appeared to eliminate the use of conventional ligands.

Acknowledgments

NJS is a National Health and Medical Research Council/National Heart Foundation of Australia C.J. Martin Overseas Research Fellow. KAB was supported by a CASE studentship from the Biotechnology and Biological Sciences Research Council (BBSRC).

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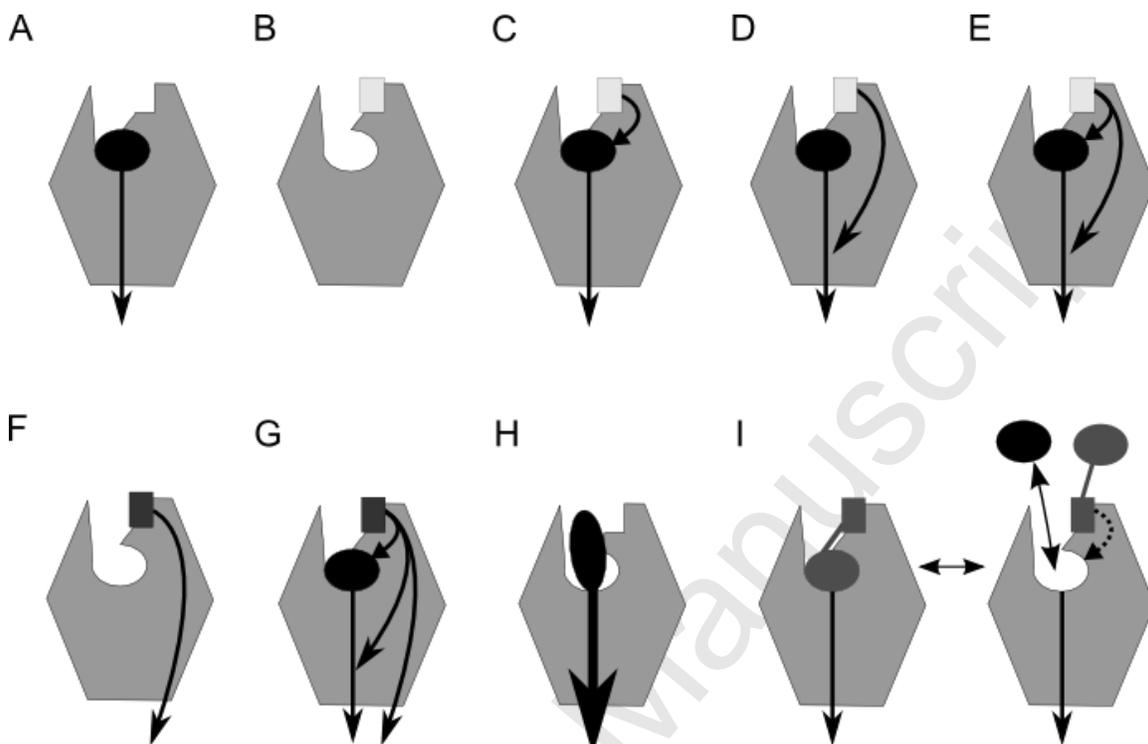
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Figure Legend

**Box 1: The expanding modes of agonism at G protein-coupled receptors**

For decades, the traditional model of receptor activation involves the interaction of a ligand at the orthosteric binding site of a receptor somewhat like a key fitting into a lock (illustrated diagrammatically in A). In the case of agonism, binding of an orthosteric agonist induces conformational changes in the receptor that result in a functional response; thus the ligand possesses efficacy. As such, an inverse agonist at the orthosteric site will act to reduce any existing function of the receptor while a neutral antagonist will occupy the binding site but will not possess efficacy. More recently, it has become apparent that compounds are able to bind and interact at a site distinct from the orthosteric site – this is referred to as the allosteric site and is represented by the rectangular ligand in diagrams (B-E). An allosteric modulator can bind to a G protein-coupled receptor

in the absence of agonist (B) without effect. However, in the presence of an orthosteric ligand (for the purposes of this review, an orthosteric agonist), an allosteric modulator can positively or negatively affect either the affinity (C), efficacy (D) or both the affinity and efficacy (E) of the orthosteric ligand.

Another possible interaction predicted by the Allosteric Two-State Model (May et al., 2007) of receptor function is allosteric agonism (F), in which case a ligand binds to an allosteric site on the receptor and causes a functional response. The allosteric agonist may also allosterically modulate the affinity and/or efficacy (as per C-E) of a ligand at the orthosteric site, in which case it is referred to as an ago-allosteric modulator (G). Less frequently, an agonist may possess efficacy above that of the endogenous ligand (so-called super-agonism; although represented here at the orthosteric site there is no reason why super-agonism could not occur via allosteric agonism or ago-allosterism). Finally, subtype selectivity can be enhanced by the design of dualsteric ligands in which a potent but non-selective orthosteric ligand is fused to a more selective moiety that interacts at the allosteric binding site (I). However, there is also the possibility that the dualsteric ligand will bind only at the allosteric site, allowing access for another ligand to bind orthosterically – the affinity and/or efficacy of the orthosteric interaction may be modulated by the allosteric moiety of the dualsteric ligand, depending upon its allosteric properties. Ideally, if the purpose of designing a dualsteric ligand is to generate an agonist with enhanced subtype specificity, then the allosteric moiety should have near-neutral co-operativity.

Although not depicted here, it is important to remember that each of the examples of efficacy above may represent full, partial or inverse agonism or even activation of subsets of the full complement of signaling pathways (functional selectivity).

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