Perspectives in Pharmacology

Allosteric Modulators of G Protein-Coupled Receptors: Future Therapeutics for Complex Physiological Disorders

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ABSTRACT

G protein-coupled receptors (GPCRs) are one of the most important classes of proteins in the genome, not only because of their tremendous molecular diversity but because they are the targets of nearly 50% of current pharmacotherapeutics. The majority of these drugs affect GPCR activity by binding to a similar molecular site as the endogenous cognate ligand for the receptor. These "orthosterically" targeted drugs currently dominate the existing pharmacopeia. Over the past two decades, novel opportunities for drug discovery have risen from a greater understanding of the complexity of GPCR signaling. A striking example of this is the appreciation that many GPCRs possess functional allosteric binding sites. Allosteric modulator ligands bind receptor domains topographically distinct from the orthosteric site, altering the biological activity of the orthosteric ligand by changing its binding affinity, functional efficacy, or

both. This additional receptor signaling complexity can be embraced and exploited for the next generation of GPCR-targeted therapies. Despite the challenges associated with detecting and quantifying the myriad of possible allosteric effects on GPCR activity, allosteric ligands offer the prospect of engendering a facile stimulus-bias in orthosteric ligand signaling, paving the way for not only receptor-selective but also signaling pathway-selective therapies. Allosteric modulators possess specific advantages when considering the treatment of multifactorial syndromes, such as metabolic diseases or age-related cognitive impairment, because they may not greatly affect neurotransmitter or hormone release patterns, thus maintaining the integrity of complex signaling networks that underlie perception, memory patterns, or neuroendocrinological axes while introducing therapeutically beneficial signal bias.

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Heptahelical GPCRs are ubiquitously expressed throughout eukaryotic organisms and can account for as much as 3 to 4% of the genome (Foord, 2002). By detecting ligands in the extracellular milieu, they transmit environmental information from outside a cell to the interior. At the same time, changes in the expression of GPCRs or receptor-associated regulatory proteins under varying physiological conditions

ABBREVIATIONS: GPCR, G protein-coupled receptor; AM, allosteric modulator; PAM, positive allosteric modulator; NAM, negative allosteric modulator; ATCM, allosteric ternary complex model; NMS, *N*-methylscopolamine; AchR, acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; mGluR, metabotropic glutamate receptor; CTC, cubic ternary complex; CB₁, cannabinoid 1; PTH, parathyroid hormone; CPPHA, *N*-(4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl) methyl] phenyl)-2-hydroxybenzamide; WIN55212, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo-[1,2,3-*d,e*]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; McN-A-343, 4-(*m*-chlorophenyl)-carbamoyloxy-2-butynyltrimethylammonium chloride; AC-42, 4-*N*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; Org27569, 5-chloro-3-ethyl-*N*-[2-[4-(1-piperidinyl)phenyl]ethyl-1*H*-indole-2-carboxamide; VU0090157, cyclopentyl 1,6-dimethyl-4-(6-nitrobenzo[d][1,3]-dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate; VU0029767, (E)-2-(4-ethoxyphenylamino)-*N'*-[(2-hydroxynaphthalen-1-yl) methyl-ene] acetohydrazide; BQCA, benzyl quinolone carboxylic acid; VU10010, 3-amino-*N*-(4-chlorobenzyl)-4,6-dimethylthieno[2,3-*b*] pyridine-2-carboxamide 2,2,2-trifluoroacetate; CP55940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; LY2033298, 3-amino-5-chloro-*N*-cyclopropyl-6-methoxy-4-methyl-thieno[2,3-*b*]pyridine-2-carboxamide; ABP-280, 280-kDa actin-binding protein.

can preprogram cells to respond in certain ways to external stimuli (Maudsley et al., 2004). GPCRs have evolved to allow cellular systems to sense their environment through selective recognition of almost every type of agent, e.g., photons, odors, lipids, amino acids, carbohydrates, and complex polypeptides. Because of this unparalleled flexibility and their involvement in most of the physiological processes in an organism, it is not surprising that GPCRs have proven to be effective pharmacological drug targets. Appreciation of the functional complexity of GPCR biology in recent years has increased exponentially. It is now accepted that GPCRs interact with many proteins to exert their full range of activities (Brady and Limbird, 2002; Maudsley et al., 2004, 2007) and that, as with other transmembrane receptors (e.g., ligand-gated ion channels), they are subject to modulation by ligands that can act independently or cooperatively with the endogenous cognate ligands. This latter aspect of GPCR signaling, i.e., allosteric receptor modulation, will be the primary subject of this review as this capacity to synergize with the endogenous agents and their more subtle mode of activity makes allosteric modulators (AMs) prototypes for the next generation of therapeutics to treat complex disorders that affect multiple aspects of peripheral and central nervous tissue function (Christopoulos, 2002; Leach et al., 2007; Lewis et al., 2008; Conn et al., 2009).

Orthosteric and Allosteric Modulation of Heptahelical GPCRs

Modern classification of heptahelical GPCRs subdivides the superfamily into five major functional groups—glutamate receptors, rhodopsin-like receptors, adhesion family receptors, frizzled/taste receptors, and secretin-like receptors (Schiöth and Fredriksson, 2005)—that collectively mediate the cellular sensation of the most environmentally derived and endogenous somatic compounds. The rhodopsin-like class of GPCRs includes some of the most studied proteins in nature. Many of the GPCRs in this class have been subjected to extensive structure-function analysis by site-directed mutagenesis. Most rhodopsin-like GPCRs possess a distinctive orthosteric binding site (i.e., domain involved in docking of the endogenous ligand with the receptor), either deep within the helical bundle for small ligands (e.g., biogenic amines) or superficially across the extracellular loops and surface helical regions for larger ligands (e.g., small neuropeptides). This orthosteric binding site facilitates high-affinity ligand binding and allows transduction of the stimulus to the interior of the cell. It has been shown for most endogenous ligands that binding to this site initiates the majority of signaling activity associated with receptor-ligand engagement. Orthosteric ligand GPCR activation classically transmits a signal, mediated by conformational rearrangement, across the plasma membrane to the intracellular domains of the receptor. By contrast, AMs do not directly engage the orthosteric site. The binding of an AM may cause a conformational change in the receptor protein that is transmitted to the orthosteric site (and vice versa), in essence creating a “new” GPCR with its own set of binding and functional properties. In addition, AMs may engender collateral efficacy by biasing the stimulus, thus leading to signaling-pathway-selective allosteric modulation (either enhancement or blockade). In the context of pharmaceutical development, AMs are generally thought

of as exogenous compounds, often small molecules that bind a region of the receptor that is distant from the native orthosteric site. However, it is important to consider that GPCRs interact with numerous intracellular proteins (e.g., heterotrimeric G proteins) that also affect receptor conformation. Thus, in the broad context, allosteric modulation of GPCR function can arise from the association of accessory proteins with the internal face of the receptor (Maudsley et al., 2005) or through AM interaction with intracellular binding sites (Espinoza-Fonseca and Trujillo-Ferrara, 2006). To understand the role of AMs in controlling GPCR responses to ligand-induced conformational changes, we shall first consider dynamic models of GPCR function.

Modeling Allosteric Modulation

In classical dynamic models of GPCR function, the receptor transmits the orthosteric ligand signal by functioning as a ligand-activated guanine nucleotide exchange factor for juxtamembrane heterotrimeric G proteins. G protein activation is initiated through ligand-driven changes in the tertiary structure of the transmembrane heptahelical receptor core (Ballesteros and Palczewski, 2001; Shapiro et al., 2002). These conformational changes are transmitted to the intracellular transmembrane loops and carboxyl terminus and alter the ability of the receptor to catalyze the rapid exchange of GDP for GTP on the heterotrimeric G protein α -subunit. The GTP-bound α -subunit then can stimulate its cognate downstream effectors, e.g., phospholipase C or adenylate cyclase, conveying information about the presence of the stimulus in the extracellular environment. In this basic conceptualization, the GPCR functions as a switch, existing in either an “off” or “on” state. Evidence that GPCR behavior is more complex originated with the finding that β -adrenergic receptors exhibit two affinity states for agonists, the relative proportions of which are modulated by the presence of guanine nucleotides (DeLean et al., 1980). The model advanced to explain these phenomena predicted that, in the presence of GDP, agonist binding promotes the formation of a long-lived ternary complex among agonist (H), GPCR (R), and heterotrimeric G protein (G) that exhibits high agonist binding affinity. In the absence of the G protein or when the presence of GTP allows for receptor-catalyzed G protein activation, the H-R-G complex is dissociated, and the receptor resides in a low-affinity (H-R) state. However, even this simplistic model accommodates a wide variety of orthosteric effects. Ligands can act as positive agonists (stimulating G protein turnover), inverse agonists (reducing constitutive G protein activation by the unliganded receptor), partial agonists (exhibiting lower intrinsic efficacy than a full agonist), or classical antagonists (binding the orthosteric site without G protein activation).

Along with the increasing complexity of orthosteric ligand-receptor interactions, the past decade has witnessed an increase in the number of potential therapeutic ligands that target GPCRs by binding to allosteric sites on the receptor. AMs may increase or decrease the ability of the orthosteric ligand to interact with the receptor and/or modulate its ability to stabilize the active conformation of the receptor. Although both modulatory processes may occur simultaneously, the most commonly observed AM effect is modulation of orthosteric ligand affinity. As with the ternary

complex model for orthosteric ligand-receptor interactions, models have been generated for AM interactions (May et al., 2004). One model designed to quantify AM activity is described as the allosteric ternary complex model (ATCM; Fig. 1A). The ATCM is the simplest mass-action scheme applied to allosteric interactions, and its properties at equilibrium have been used to derive quantitative models for AM activity simulation (Stockton et al., 1983; Ehlert, 1988; Christopoulos and Kenakin, 2002). The ATCM can be used to quantify AM activity in terms of ligand affinity for the unoccupied receptor and its cooperativity factor (α). The cooperativity factor is a thermodynamic measure of the strength and direction of the allosteric change in affinity for one site when the other is occupied. Allosteric modulators can be broadly grouped as either positive AMs (PAMs, $\alpha > 1$) or as negative AMs (NAMs, $\alpha < 1$). For example, the binding of the orthosteric antagonist *N*-methylscopolamine (NMS) to the M_2 -muscarinic acetylcholine receptor (mAChR) is allosterically enhanced by alcuronium (Avlani et al., 2004) but is allosterically inhibited by gallamine, even though both AMs bind to a common allosteric site on the receptor (Lanzafame et al., 1997).

Beyond effects on orthosteric ligand affinity, AMs can produce changes in the intrinsic efficacy of the receptor-orthosteric ligand complex. This property is exemplified by a series of modulators of cannabinoid CB_1 receptors. The allosteric modulator Org27569 enhances the binding of the orthosteric agonist CP55940 at mouse CB_1 receptors but significantly reduces the efficacy of the orthosteric agonist WIN552122 for inhibition of electrically evoked contractions in a mouse vas deferens preparation and the efficacy of CP55940 at human CB_1 receptors in a reporter-gene assay (Price et al., 2005). To accommodate these effects, an allosteric two-state model has been proposed that provides an additional cooperativity factor governing the transition of the receptor between a resting (R) and an activated (R^*) state in the presence of an allosteric ligand, the allosteric cubic ternary complex (CTC) model (Fig. 1B).

Although most allosteric GPCR modulators are pharmacologically quiescent in the absence of an orthosteric ligand, it

has been noted that some allosteric ligands, termed “ago-allosteric” modulators, act as agonists in their own right (Knudsen et al., 2006). Such “allosteric agonists” further expand the number of possible receptor-ligand interactions because they have the potential to modulate orthosteric ligand pharmacology in addition to perturbing cellular signaling in their own right. Two mAChR ligands suggested to act this way are the functionally selective partial agonists, McN-A-343 and AC-42. In addition to engendering partial agonist effects, they produce incomplete inhibition of the binding of the orthosteric antagonist NMS when present at saturating concentrations at rat M_2 (McN-A-343) and human M_1 (AC-42) mAChRs while retarding NMS dissociation (Langmead et al., 2006; Valant et al., 2008). It is noteworthy that it is also possible for a ligand to bind to an allosteric site without altering orthosteric regulation of receptor function, in effect acting as a “neutral” antagonist at the allosteric site.

Allosteric Receptor Modulation by GPCR Accessory Proteins

GPCRs are naturally allosteric proteins that interact with numerous other proteins that alter their ligand-binding affinity or signaling properties. In effect, heterotrimeric G proteins are AMs, in that they alter ligand affinity by contacting the receptor at a topographically distant site from the orthosteric binding site. Numerous other proteins interact with the intracellular face or transmembrane regions of GPCRs. GPCR-interacting proteins include kinases (e.g., G protein-coupled receptor kinases and protein kinase A) (Fraser et al., 2000), arrestins (Pfister et al., 1985), the 4.1 family of cytoskeletal proteins (e.g., ABP-280) (Li et al., 2000), amyloid precursor like protein 1 (APLP1) (Weber et al., 2006), receptor activity-modifying proteins/calcitonin gene-related peptide-receptor component protein (McLatchie et al., 1998; Evans et al., 2000), and PDZ-domain containing proteins (Hall et al., 1998). These interacting proteins influence GPCR signaling by regulating downstream effectors and by participating in scaffolding, endocytosis, trafficking, or recy-

Fig. 1. The basic and cubic allosteric ternary complex mechanisms. **A**, the ATCM, a concise framework for modeling the interaction of two ligands (e.g., an orthosteric agonist A and an allosteric modulator, B) on a receptor, in terms of their respective equilibrium dissociation constants (K_A and K_B), and a cooperativity factor, α , that denotes the magnitude and direction of the allosteric effect on ligand binding affinity. Stimulus is assumed to be imparted to the cell by the orthosteric agonist binding receptor (AR) and the modulator-orthosteric agonist binding species (ARB), and an additional proportionality factor, β , may be added to account for modulator-induced alterations in efficacy. **B**, the allosteric CTC model. This model allows the active state to interact with G protein and the inactive R^* state. This model is formally identical with the allosteric two-state model of Hall (2000), which describes the interaction of an allosteric modulator and orthosteric ligand on a receptor that can adopt active and inactive conformations. The terms β , γ , and δ (for the CTC model) are ligand-related and describe the change in the receptor affinity, for the G protein, imparted by the ligand.

cling of the receptor (Engström et al., 2006; Tobin, 2008). For example, the interaction of the vasoactive intestinal polypeptide-pituitary adenylate cyclase-activating peptide receptor (VPAC1R) with the accessory protein receptor-activity-modifying protein 2 enhances $G_q/11$ -mediated phosphoinositide accumulation while leaving receptor coupling to the G_s -coupled cAMP response essentially unaltered (Conner et al., 2005). Another example is the binding of neurochondrin to the C-terminal tail of the melanin-concentrating hormone receptor (MCHR1), which inhibits agonist stimulation of $G_{i/o}$ and $G_{q/11}$ signaling but not agonist-induced internalization (Francke et al., 2006). In principal, sites of interaction with accessory proteins are potential points at which GPCRs can be allosterically manipulated, and any agent that targets one of these accessory proteins or their site of interaction with the receptor could be considered a functional AM.

A compelling example of such “indirect” allosteric modulation involves the regulation of GPCR heterodimers, wherein an orthosteric or allosteric ligand for one receptor modulates signaling of the dimer partner through conformational changes transmitted by contact between receptor transmembrane domains. It is now clear that most, if not all, GPCRs are able to form oligomers as either homodimers or heterodimers with other GPCRs. Dimerization affects many aspects of GPCR function, including subcellular trafficking and signaling. The first widely accepted demonstration involved the metabotropic GABA_B receptor, which functions as an obligate heterodimer. The receptor is composed of two isoforms GABA_B-1 and GABA_B-2 that are nonfunctional when expressed individually but become functional when coexpressed, given that heterodimerization is required for post-translational trafficking to the plasma membrane (Jones et al., 1998; Kaupmann et al., 1998; Ng et al., 1999). Receptor heterodimerization also affects ligand binding. For example, positive cooperativity has been reported for the ligand binding of δ - and κ -opioid receptors when coexpressed (Jordan and Devi, 1999). Conversely, negative cooperativity in dopamine D₂ receptor agonist binding in the presence of an adenosine A₂ receptor agonist has been observed when the receptors were coexpressed (Franco et al., 2000). In practical terms, this means that the orthosteric ligand binding site of one receptor acts as an allosteric site for the heterodimer partner. In the context of μ - δ -opioid receptor dimers, antagonist occupancy of δ receptors enhances μ -opioid receptor agonist binding and signaling *in vitro*, and δ -opioid antagonists enhance morphine-induced analgesia *in vivo* (Gomes et al., 2004). Allosteric antagonism of GPCR heterodimers is also possible. In murine cardiomyocytes, antagonism of β -adrenergic receptors inhibits angiotensin AT1a receptor-mediated contractility and vice versa (Barki-Harrington et al., 2003). This phenomenon arises within β 2-adrenergic/AT1a receptor heterodimers, wherein each receptor is uncoupled from its cognate G proteins when its heterodimer partner is bound to an orthosteric antagonist. Such a requirement for dual receptor occupancy may be a common phenomenon. For example, the interaction of M₃AChR dimers with β -arrestin-1 and the subsequent activation of mitogen-activated protein kinase requires agonist binding to each receptor protomer (Novi et al., 2005).

Given the evidence that GPCRs form hetero-oligomers with unique ligand-binding and signaling properties, it is tempting to hypothesize that AMs might influence GPCR signaling in a multitude of ways, from changing ligand affinity/selectivity

to altering downstream coupling preference or targeting other protomer receptors or accessory proteins to the complex. Allosteric regulation within di(oligo)mers implies that the pharmacological properties of a given receptor subtype can be influenced by the array of dimerization partners co-expressed in each particular cell type (George et al., 2000). Future work will be required to identify, at the molecular level, the conformational changes involved in these allosteric interactions, explain how agonists and antagonists exert positive and negative cooperative effects on dimer partners, and how these allosteric effects can be exploited to change the “texture” of these complex and diverse GPCR signaling states.

Allosteric Modulation of GPCR Functional Selectivity

Despite their utility in describing orthosteric signaling, it is now clear that two-state models cannot predict the full range of orthosteric ligand effects. Many ligands that behave as inverse agonists or classical “neutral” antagonists for one effector pathway have been found to exert opposing effects, e.g., agonist or partial agonist activity, for receptor coupling to an alternative G protein or G protein-independent pathway (Maudsley et al., 2000; Zhang and Neer, 2001; Maudsley et al., 2004). The phenomenon of ligand coupling a receptor to only a subset of its potential effectors is known by several different terms, including functional selectivity, agonist-directed trafficking of receptor stimulus, biased agonism, differential engagement, and stimulus trafficking (Berg et al., 1998; Bonhaus et al., 1998; Brink et al., 2000; MacKinnon et al., 2001; Kenakin, 2002, 2003).

The linkage between functional selectivity and receptor conformational state has been known for at least a decade. The phenomenon of reversal of potency, wherein a series of orthosteric agonists interacting with the same receptor exhibit different rank orders of potency when compared by using two or more readouts of receptor activation, implies that GPCRs can exist in more than one “active” conformation (Perez et al., 1996; Palanche et al., 2001; Galandrin and Bouvier, 2006). Evidence of the existence of distinct active receptor conformations has come from multiple sources using a wide array of receptor paradigms (Mundell et al., 1997; Maudsley et al., 1998; Kohout et al., 2004) and biophysical techniques (Ghanouni et al., 2001; Audet et al., 2008). The recent discovery of G protein-independent signals, e.g., transmitted by arrestin-bound signaling proteins (Luttrell et al., 1999), has led to even more dramatic examples of ligands that exhibit true reversal of efficacy. For example, the parathyroid hormone (PTH) analog, [D-Trp12, Tyr34]-PTH(7–34), acts as an inverse agonist for PTH1 receptor coupling to G_s -adenylyl cyclase and has no intrinsic efficacy for $G_q/11$ -coupling but behaves as an agonist with respect to arrestin recruitment, receptor internalization, and arrestin-dependent extracellular signal-regulated kinase activation (Gesty-Palmer et al., 2006). Such behavior can only be modeled on the basis of multiple active receptor conformations and underscores the potential for pharmacologically biasing signal output.

AM binding may also affect receptor conformation to favor certain active states or change the interaction of the receptor with other juxtamembrane or transmembrane proteins, biasing the signal output generated by endogenous orthosteric

ligands or even creating new “flavors” of receptor with unique functional properties (Maudsley et al., 2005). For example, in cortical astrocytes, an AM of the metabotropic glutamate receptor (mGluR) type 5, *N*-[4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl) methyl] phenyl]-2-hydroxybenzamide (CPPHA), potentiates calcium mobilization by the orthosteric agonist 3,3'-difluorobenzaldazine but decreases the maximal extracellular signal-regulated kinase activation stimulated by the same orthosteric agonist (Zhang et al., 2005). Signaling pathway-selective effects could also enable AMs to generate “collateral” efficacy, offering the potential for “selecting” desired pharmacological effects and excluding undesired effects by targeting allosteric sites. Thus, the binding of the PAM peptide ASLW to the CXCR4 chemokine receptor induces a stronger chemotactic immune cell response than the orthosteric ligand, CXCL12, but does not promote receptor internalization like CXCL12 (Sachpatzidis et al., 2003).

Designing Allosteric Modulators

Unlike orthosteric agents, the discovery of AMs has primarily been through the use of functional assays. Because many allosteric recognition sites lie outside of the classical orthosteric regions, the recent elucidation of higher resolution molecular GPCR structures and refined structural models (Rasmussen et al., 2007) is likely to aid the design of the next generation of AMs. Using such high-resolution crystal structures, the commonalities of AM binding pockets may become more apparent. However, there are specific considerations that are pertinent to the discovery process for AMs, e.g., the correct attribution of insurmountable drug effects to orthosteric or allosteric effects using well defined experimental criteria (Kenakin et al., 2006). An improved molecular dynamic understanding of the true range of functional activities of GPCRs via the use of techniques such as bioluminescence resonance energy transfer (Angers et al., 2000), optical dynamic mass redistribution (Kebig et al., 2009), and GPCR cellular impedance assays (Peters and Scott, 2009) will undoubtedly also prove valuable for future AM design. In recent years, significant progress in the rational design of AM agents has been made, especially for muscarinic acetylcholine, metabotropic glutamate, adenosinergic, and GABAergic receptors. Past attempts at therapeutic targeting of muscarinic acetylcholine receptors with orthosteric agents have largely failed due to poor receptor subtype selectivity. Recently, multiple AMs displaying excellent subtype selectivity for M₁ muscarinic receptors have been developed, as well as two agents for the M₄ subtype (Lazareno et al., 2004; Shirey et al., 2008). Perhaps paving the way for future AM design, the latter M₄-selective agent, VU100010, was designed from a pre-existing compound (LY2033298) from Eli Lilly and Company (Indianapolis, IN) using a medicinal chemistry-chemoinformatic hybrid approach.

The ability to predict structure-activity relationships in AMs is in its infancy, although recent work with metabotropic glutamate receptors has begun to provide insight. Recently developed AMs for mGluR1 (Knoflach et al., 2001; Zheng et al., 2005; Wu et al., 2007), mGluR2 (Johnson et al., 2005), mGluR4 (Stachowicz et al., 2004), and mGluR5 (Lindsley et al., 2004; Roppe et al., 2004; Kinney et al., 2005; Porter et al., 2005; Zhao et al., 2007) have shown in vitro or clinical efficacy for chronic pain, anxiety, Parkinson's disease, and schizophrenia. Several of these series, most notably the PAMs (–)

PHCCC (mGluR4) (Stachowicz et al., 2004) and CPPHA (mGluR5) (Zhao et al., 2007) demonstrate a surprisingly sensitive structure-activity relationship, because even a slight modification of the parent compound almost invariably results in inactive derivatives. Whether such features are characteristic of PAMs compared with NAMs, in general, remains to be seen, but these distinctions may be indicative of a qualitative difference in the pharmacological nature of the dynamic relationship of PAM interaction with GPCRs compared with NAMs. From this finding, one could posit that there may be greater molecular diversity for NAMs and therefore a greater number of potential NAM binding sites on GPCRs.

The sheer molecular diversity of AMs adds an additional layer of complexity to compound design and screening programs. Unlike orthosteric ligands, which are structural mimetics of endogenous compounds that evolved to confer fidelity in receptor activation, AMs have much greater potential to produce off-target effects via receptor or nonreceptor interactions. There are many examples of allosteric modulating agents that possess other significant pharmacological functions. Although its therapeutic effect results from blockade of epithelial sodium channels, the diuretic amiloride also exerts an allosteric effect at α 2A/2B adrenergic receptors (Leppik and Birdsall, 2000). The cyclooxygenase inhibitor salicylic acid not only acts via modulation of the prostanoid system, it also allosterically modulates the endothelin A receptor (Talbot et al., 2000). The endogenous soporific lipid, oleamide, is both an endogenous cannabinoid CB1 receptor agonist and a potent allosteric modulator of multiple serotonin receptor subtypes (Thomas et al., 1997). The greater potential for cross reactivity inherent in small molecules needs to be considered both when screening AMs for activity and when attempting to ascribe physiologic effects on complex systems to a specific receptor target.

Allosteric Modulators in the Clinic

The two AMs that have been approved to date for clinical use illustrate the diverse effects attainable through allosteric modulation of GPCR function. The first to enter the market was Cinacalcet, a PAM of the calcium sensing receptor (CaSR) approved for treatment of secondary hyperparathyroidism and parathyroid carcinoma (Goodman et al., 2002; Nemeth et al., 2004; Szmulowicz and Utiger, 2006). In chronic kidney disease, loss of renal 1 α hydroxylation of [25-OH]-vitamin D2 impairs intestinal calcium absorption, leading to chronic hypocalcemia. This reduces tonic inhibition of PTH secretion by the CaSR in the parathyroid glands, leading to excessive PTH secretion. Elevated levels of PTH, the hallmark of secondary hyperparathyroidism, are associated with altered metabolism of calcium and phosphorus, bone pain, fractures, and an increased risk for cardiovascular death. Cinacalcet increases CaSR affinity for calcium, and effectively suppresses PTH secretion despite hypocalcemia. Conversely, Maraviroc (UK-427, 857) is a noncompetitive allosteric antagonist of the chemokine receptor, CCR5, that was recently approved as salvage therapy in advanced HIV disease (Wood and Armour, 2005). CCR5 acts as the cell surface coreceptor for the HIV virus. Maraviroc binding to CCR5 alters receptor conformation so as to block HIV bind-

ing, reducing HIV infectivity and producing a marked fall in systemic viral load (Rosario et al., 2008).

Allosteric Modulators in the Treatment of Complex Disorders

Over 90% of nonsensory GPCRs are expressed in the brain, where they regulate myriad neuronal and endocrine functions. Exploiting GPCR pharmacology to develop treatments for age-related conditions such as dementia will require a more advanced understanding of the complex changes in neuroendocrine interactions that occur with the aging process (Martin et al., 2008). Because of the immense complexity and interconnectedness of central nervous system signaling networks it is likely that therapeutic agents will need to produce highly specific and perhaps subtle changes. It is in these areas that the pleiotropic nature of AM actions offer potential advantages over orthosteric ligands (Fig. 2).

One significant barrier to orthosteric drug design is the difficulty of achieving selective drug targeting to well conserved and singular orthosteric binding regions. Although GPCRs respond to the widest range of compounds of any 'receptive' biological system, most are clustered into groups of closely related receptors that share a common endogenous ligand. The muscarinic M_1 – M_5 receptors, metabotropic mGlu1–mGlu8 receptors, and the approximately 13 members of the serotonin 5-HT receptor family exemplify this property. While selectivity between families of receptors that bind structurally distinct ligands is usually achievable (e.g., a compound that interacts with mGluRs but not 5-HT receptors), it is often difficult to obtain subtype selectivity among members of an individual family using orthosteric ligands. A high degree of conservation in the amino acid sequences

coding for the orthosteric binding site across all subtypes within a receptor family often precludes discovery of highly selective compounds. Despite much effort by medicinal chemists, a number of traditional orthosteric agonists of the muscarinic acetylcholine receptor evaluated in clinical trials for the treatment of Alzheimer's disease, including milameline, sabcomeline, cevimeline, and talsaclidine, have all shown therapeutic efficacy but ultimately failed because of poor subtype selectivity and associated side effects. In contrast, AMs can exhibit exquisite selectivity between closely related receptors (Lazareno et al., 1998; Ellis and Seidenberg, 2000). One reason for this may be that allosteric sites are placed under less evolutionary pressure with respect to conservation of function and thus display wider protein sequence divergence across receptor subtypes relative to orthosteric sites (Bridges and Lindsley, 2008). For example thiochrome binds to an allosteric site on all five subtypes of mAChR, but selectively enhances the affinity of acetylcholine only at the M_4 AChR because the cooperativity between the orthosteric and the allosteric sites is neutral ($\alpha = 1$) at all other subtypes (Lazareno et al., 2004). Likewise, agents such as the M_1 / M_4 AChR allosteric agonist xanomeline and the M_1 AChR allosteric agonists AC-42, *N*-desmethyloclozapine, and 1-(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1*H*-benzo[d]imidazol-2(3*H*)-one have displayed unprecedented subtype selectivity (Spalding et al., 2002; Mirza et al., 2003; Sur et al., 2003; Jones et al., 2008).

In addition to these novel M_1 -selective allosteric agonists, exciting progress has been made in the discovery of novel PAMs of the M_1 AChR. For instance, VU0090157, VU0029767, and BQCA were recently identified in a high-throughput functional screening program (Marlo et al., 2009). None of these

Fig. 2. Multiplicity of activities of GPCR-targeted allosteric modulating agents. There are multiple functional mechanisms thought to underlie the pleiotropy of AM. Compared with conventional orthosteric ligands, AMs may be more sensitive to subtle alterations in GPCR function induced by receptor dimerization (1) or interaction with intracellular scaffolding proteins (2). This sensitivity may influence the signal-conditioning effects of the AM. The identification of multiple extracellular or intracellular functional binding sites for AMs on GPCRs (3) lends the capacity to target site-specific compounds based on their ability to partition into the plasma membrane. Not only can AMs modify direct stimulatory signaling processes induced by the orthosteric ligand but they may also control the directionality of tachyphylactic mechanisms as well as homologous desensitization (4).

M₁AChR PAMs had agonist activity or competed for binding at the orthosteric acetylcholine binding site. However, each induced parallel leftward shifts in acetylcholine affinity, indicating that they enhance M₁AChR activity by increasing affinity for the orthosteric ligand. Furthermore, VU0090157 produced equivalent potentiation of M₁AChR activation of phospholipase C and phospholipase D activity, whereas VU0029767 enhanced phospholipase C, but not phospholipase D, signaling. This illustrates another potential advantage of AMs, the ability to bias receptor output by differentially regulating receptor coupling to downstream pathways. As with VU0090157 and VU0029767, BQCA has no activity at M₂, M₃, or M₄AChRs. In a contextual fear-conditioning model of episodic-like memory, BQCA fully reverses the cognitive impairment induced by scopolamine through a possible cognition-enhancing effect (Marion et al., 2008; Marlo et al., 2009). These findings suggest that it will be possible to develop highly selective M₁AChR PAMs with potential benefit in cognitive disorders. Other subtype-selective AMs have shown promise in modifying cortical brain function. LY2033298 is a highly selective PAM of the M₄AChR that has shown significant efficacy in preclinical models predictive of antipsychotic drug behavior (Chan et al., 2008). In addition, the mGluR5 potentiator 3,3'-difluorobenzaldazine is the first highly selective allosteric activator of mGluRs. Its discovery stimulated further efforts leading to the identification of other series of mGluR5 potentiators that have now been shown to have antipsychotic-like activity in animal models (Maj et al., 2003; Marino et al., 2003; Kinney et al., 2005).

Allosteric modulators, with little inherent intrinsic activity that act by enhancing or attenuating the response elicited by the endogenous transmissive compound, offer several potential advantages over conventional agonists and antagonists in treating complex conditions and syndromes. First, AM effects are often saturable and therefore less likely to elicit adverse effects from overdose. Second, their effects are often exerted primarily in the presence of the native orthosteric ligand. Thus, AM activity is tied to the temporal pattern of synaptic signaling or endocrine hormone release, such that they only amplify or reduce the receptor signal when the hormone or neurotransmitter is released. Hence, an AM will preserve the physiological receptor signaling hierarchy in complex neuroendocrine axes and neurotransmissive events, while at the same time, boosting the efficiency of the endogenous neurotransmitter/hormone. Furthermore, a lack of chronic receptor activation may cause less receptor desensitization or internalization over time, overcoming the problem of diminishing therapeutic efficacy that is seen with many chronically administered orthosteric agonists. This could prove especially important in the case of neurodegenerative diseases such as Alzheimer's disease where decreased levels of acetylcholine in the forebrain impair cognition (Hasselmo and Giocomo, 2006; Fisher, 2008) or in complex endocrine disorders such as diabetes (Martin et al., 2008, 2009). Third, AMs can bias signal output in favor of only part of the receptor response profile. This results from conformational constraints placed on the receptor that limit its ability to engage effector/accessory proteins, e.g., GRKs or arrestins, as well as G proteins. This property is well known for orthosteric ligands, such as morphine, which promote G protein coupling without causing arrestin-dependent desensitization. The physiological responsiveness to psychostimulants and morphine suggests the involvement of

these other GPCR regulators in neurological/pathological states, such as addiction, Parkinson's disease, aging, mood disorders, and schizophrenia (Jacoby et al., 2006; Eglen et al., 2007). With high receptor subtype-specificity and functional selectivity, AMs may be better able to dissociate therapeutic and side effects, e.g., the hypotensive and sedative properties of α 2A-adrenoceptors (Kukkonen, 2004).

Finally, AMs may be able to "reprogram" cellular responses to GPCR activation. During aging, it is highly likely that both the direct receptor system and its regulatory mechanisms become disrupted and modified by accumulated changes in the transcriptome and functional proteome. Restoring normal function may be difficult using simple orthosteric pharmacology, but AMs that selectively modify receptor interactions with their effectors or regulators may restore balance or even establish new functional receptor systems with unique signaling capability.

Conclusions

In recent years, the concept of allosteric modulation of GPCRs has matured and now represents an increasingly viable approach to drug discovery and development. This is evident in the fact that AMs have been reported and designed for many types of rhodopsin-like GPCRs, and several are currently in clinical trials, with two drugs already in clinical use. While still a relatively new concept in GPCR pharmacology, AMs have the potential to provide a remarkable precision in the targeting of drugs to closely related GPCR subtypes and engendering stimulus-bias in orthosteric ligand signaling, opening up new avenues for not only receptor-selective but also signaling-pathway-selective therapies. Many aspects of allosteric receptor pharmacology lend themselves to the treatment of pathological states that affect large complicated biological systems that possess multiple levels of activity of the same hormone or neurotransmitter, e.g., neurotransmissive memory patterns or multi-organ endocrine axes feedback loops.

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