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REVIEW

G-Protein-Coupled Receptors: From Classical Modes of Modulation to Allosteric Mechanisms

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ABSTRACT Heterotrimeric G-protein-coupled receptors (GPCRs) represent a large protein family responsible for mediating extracellular to intracellular signaling within a broad range of physiological contexts. Various conventional models have been used to describe their interactions with ligands and G-proteins. In recent years, however, numerous novel ligand−receptor interactions not adequately addressed by classical receptor theory have been recognized. In addition to traditional orthosteric ligands, many GPCRs can bind allosteric ligands that modulate receptor activity by interacting with distinct or overlapping receptor sites. Such ligands include positive allosteric modulators, which have become the focus of pharmaceutical drug discovery programs and have gained the attention of a growing body of basic and translational researchers within the academic community. Here, we review the fundamental aspects of allosteric GPCR modulation by small-molecule ligands, with particular focus on the emerging position of positive allosteric modulators in modern drug discovery.

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G-protein-coupled receptors (GPCRs) represent nearly half of the current market for therapeutic agents, constitute annual revenues in excess of $40 billion, and remain a primary focus of many biomedical research and pharmaceutical drug discovery programs. Despite efforts by multiple laboratories over several decades, the crystal structures of only two GPCRs (the bovine rhodopsin and human β_2-adrenergic receptors) have been solved definitively (*1*–*4*). Since the initial report on rhodopsin, many homology models for diverse GPCRs were constructed based on this crystal structure. The first report from Koblika and coworkers (*2*) described crystallization of the β2-adrenergic receptor in a lipid environment bound to an inverse agonist affording low resolution (3.4–3.7 Å). Subsequent reports with engineered human β2-adrenergic receptor refined resolution down to 2.4 Å, which demonstrated significant differences between rhodopsin and the β2-adrenergic receptor, highlighting the challenges and cautions of employing rhodopsin alone as a template model for such a large receptor family (*3*, *4*). These new crystal structures provide exciting opportunities for analyzing orthosteric and allosteric binding sites as well as redefining GPCR structure. However, much is known about the basic structure and function of many GPCRs because of decades of biochemical, genetic, imaging-based, and molecular pharmacological research. Here, we first review the fundamental aspects of GPCRs in terms of their general structure and functional characteristics.

GPCRs comprise a diverse family of integral membrane proteins that are responsible for conveying extracellular signals to the inside of the cell *via* interactions with intracellular heterotrimeric G proteins, which in turn affect enzymes, ion channels, and other intracellular messengers. Nearly a thousand GPCRs exist, mediating

a host of molecular physiological functions by serving as receptors for hormones, neurotransmitters, cytokines, lipids, small molecules, and various sensory signals (such as light and odors), to name a few. All GPCRs possess seven transmembrane helices, three extracellular loops, and three intracellular loops, with an extracellular N-terminal tail and an intracellular C-terminal tail (Figure 1). The heptahelical transmembrane domain is largely hydrophobic, whereas the extracellular (e_1-e_3) and intracellular (i_1-i_3) segments, or loops, are generally hydrophilic, as would be anticipated for amino acids exposed to the phospholipid-rich membrane and the water-rich environments, respectively. The seven transmembrane helices are each ~24 amino acids long, while the C- and N-terminal tails as well as the loops can vary widely in length with up to hundreds of amino acids. For example, the metabotropic glutamate receptors possess N-terminal segments in the 600 amino acid range (*5*). On the basis of sequence homology and functional roles, GPCRs can be divided into five or sometimes six families, although the most common division is into three main families (or classes): A, B, and C (Figure 2). The families are readily distinguished by comparing their amino acid sequences, wherein Family B is characterized by a large extracellular loop and Family C has a large, bilobed extracellular Venus-flytrap-like domain. A second major difference between the families concerns the location of the orthosteric binding site and the nature of the orthosteric ligand. As shown in Figure 2, the orthosteric binding domain (OBD) of Family A GPCRs is located with the 7TM domain, whereas the OBD is located in the large extracellular loop within Family B and within the extracellular Venus-flytrap-like domain in Family C.

According to traditional two-state models of receptor theory, GPCRs can be conceptualized as operating in equilibrium between two functional conformations, an active (R*) and inactive (R) state (*6*). In the R* state, the receptor has higher affinity for G-proteins, which normally exist apart from the receptor as a GDP-bound Gαβγ heterotrimer in their inactive form. Ligand binding to the receptor alters the equilibrium, with agonists shifting it toward the R* state, inverse agonists shifting it toward the R state, and antagonists prevent-

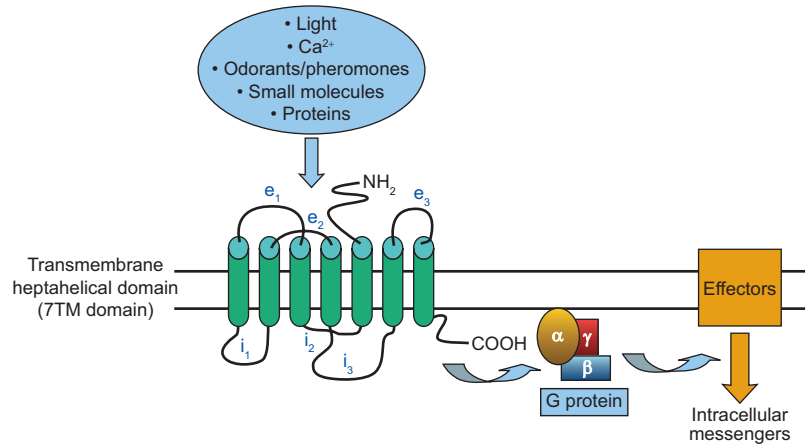

Figure 1. Representative structure of a generic GPCR. GPCRs all have a common core composed of seven transmembrane helices (the 7TM domain composed of TM-I–TM-VII) with an extracellular N-terminal domain and an intracellular C-terminal domain. The TMs are connected by three extracellular loops (e_1-e_3) and three intracellular loops (i_1-i_3). The GPCR receives an extracellular stimulus (light, calcium, odorants, pheromones, small molecules, proteins) that induces a conformational change in the receptor that either facilitates or inhibits the coupling of the receptor to a G-protein, composed of α-, β-, and γ-subunits. The G-protein, in turn, interacts with a diverse group of effectors that control intracellular messengers.

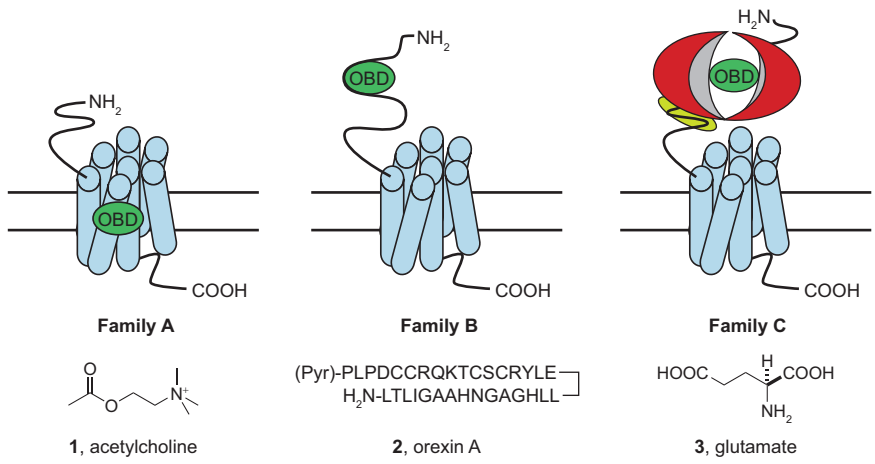

Figure 2. Representative structures of the three families of GPCRs, Family A, Family B, and Family C. Note the location of the OBD varies for the families, as does the structure of the extracellular domain. The nature of the orthosteric ligand also varies across GPCR families. For Family A, a prototypical native agonist is acetylcholine (1), for Family B, a large 33-amino acid peptide such as orexin A (2), and for Family C, glutamate (3) is a representative native ligand.

TABLE 1. Abbreviated list of the primary downstream effectors of each of the main G-protein subunits

G-protein subunit	Effector target(s)
Gβγ	Ion channels, GIRK, PI3K, phospholipases, adenylyl cyclase
$G\alpha_s$	Adenylyl cyclase (+cAMP), Na^+ and Cl^- channels
$G\alpha_{i/o}$	Adenylyl cyclase (−cAMP), K^+ and Cl^- channels, phospholipases
$G\alpha_{q/11}$	Phospholipase Cβ

ing other ligands (such as endogenous agonists) from binding without altering the basal R*:R equilibrium. Upon receptor activation, the GDP-bound G-protein interacts with the intracellular face and C-terminus of the receptor, inducing GDP to GTP exchange on the Gα subunit and concurrent dissociation of the Gα and Gβγ subunits. The now active GTP-Gα and Gβγ subunits then bind to their respective downstream effectors, which include kinases, phosphatases, small GTPases, integral membrane proteins, and a multitude of additional targets and signaling cascades. These downstream effectors exist in complex regulatory networks that control cellular functions such as movement, metabolism, membrane potential, neurotransmitter release, and gene expression. Although the simple two-state model has historically provided a highly useful framework for describing and conceptualizing receptor activity, it has been greatly expanded and modified over time as more detailed and complex ligand−receptor phenomena have emerged. As discussed in more depth below, current receptor theory models have grown to encompass a vast number of pharmacological scenarios, and these models currently play instrumental roles in the modern understanding and quantification of ligand−receptor interactions.

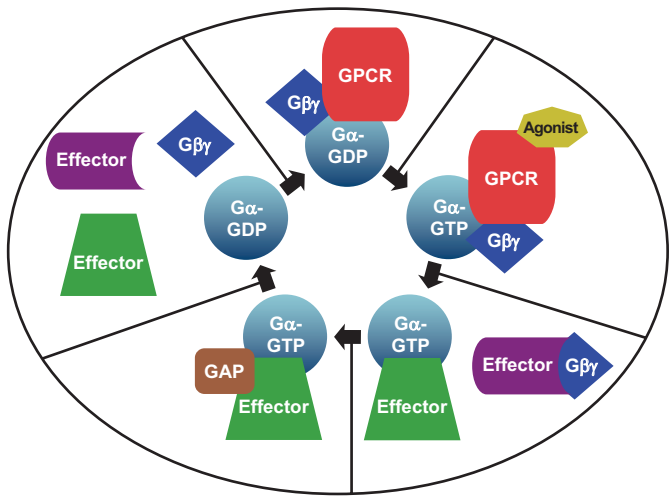

Figure 3. Generalized diagram of the G-protein cycle. Upon agonist activation of the receptor, GTP binds to the Gα subunit, displacing GDP, which causes dissociation of the protein complex from the receptor, allowing respective effector activation by Gα-GTP and Gβγ. GAPs then bind Gα and accelerate hydrolysis of GTP to GDP, which deactivates Gα and causes disengagement of the effector. Finally, Gα reassociates with Gβγ, marking cycle completion.

The specific effectors influenced by a given GPCR depend on the type of G-protein that the receptor activates. There are many types of Gα, Gβ, and Gγ subunits, allowing for diverse combinations, although the most commonly used simple categorization of GPCRs is by designation of coupling to either Gαq, Gαi, or Gαs (*7*). The mutual effector for both Gαi and Gαs is adenylyl cyclase (AC), which resides on the inner leaflet of the plasma membrane and generates cyclic-AMP in response to stimulation or inhibition by Gαs and Gαi, respectively. The primary effector for Gαq by contrast is phospholipase Cβ, a membrane-bound enzyme that converts phosphatidylinositol-4,5-bisphosphate into diacylglycerol and inositol-1,4,5-trisphosphate. Table 1 provides a limited list of the effectors for each Gα subunit. Following effector binding, the GTP-Gα subunit hydrolyzes its γ-phosphate by augmentation of its intrinsic GTPase activity *via* binding of GTPase activating proteins (GAPs), resulting in conversion to GDP-Gα. This GDP-bound form possesses higher affinity for its Gβγ subunit partner, which causes reformation of the inactive heterotrimer, marking completion of the G-protein activation cycle. Figure 3 depicts the cycle in this simplified form. Many accessory proteins and lipids are also involved in regulating G-proteins, which play important roles in controlling the G-protein cycle (*7*).

Classical GPCR ligands modulate receptor signaling by directly stimulating a receptor response (agonism), blocking the binding of the native agonist (competitive antagonism), or blocking constitutive activity (inverse agonism) of the GPCR. Classical ligands exert their modulatory effects by interacting with the orthosteric

binding site and have typically been characterized using orthosteric radioligand binding methods. GPCR signaling is also substantially influenced by receptor expression, desensitization, and internalization in response to binding by different ligands and within various cellular contexts (*8*). A major issue for GPCR agonist drugs that require chronic administration is receptor desensitization, downregulation, and/or internalization over time. By effectively turning the receptor "on" in an unnatural context, the receptor may lose sensitivity to the agonist and/or be internalized into the cell, where it is no longer available to receive extracellular stimuli. Perhaps the most widely used example of such regulation is the desensitization and internalization of the β_2-adrenergic receptor (β_2-AR) in response to chronic activation by the agonist isoproterenol (*9*). This involves binding of the β_2-AR by β-arrestin, a widely expressed cytoplasmic regulatory protein that binds GPCRs following receptor phosphorylation by GPCR kinases. Once bound, β-arrestin blocks interaction between the receptor and G-proteins, uncoupling the signaling mechanism. β-arrestin can also induce receptor internalization, reducing the pool of receptors available to ligand binding and thus diminishing signaling to downstream effector pathways (*10, 11*). Although the role of arrestins in receptor internalization and trafficking appears somewhat ubiquitous, there are arrestin-independent mechanisms as well. Furthermore, tight coupling to G-proteins is in some cases absent or secondary to signaling *via* alternative pathways (*12*). An example of such non-G-protein-mediated signaling is the direct activation of ERK2 by the β_2-AR following arrestin binding (*13, 14*). The emerging details and importance of such noncanonical signaling by GPCRs are areas of growing interest and investigational focus because such signaling is likely to underlie many interesting and underappreciated context-specific physiological mechanisms and functions.

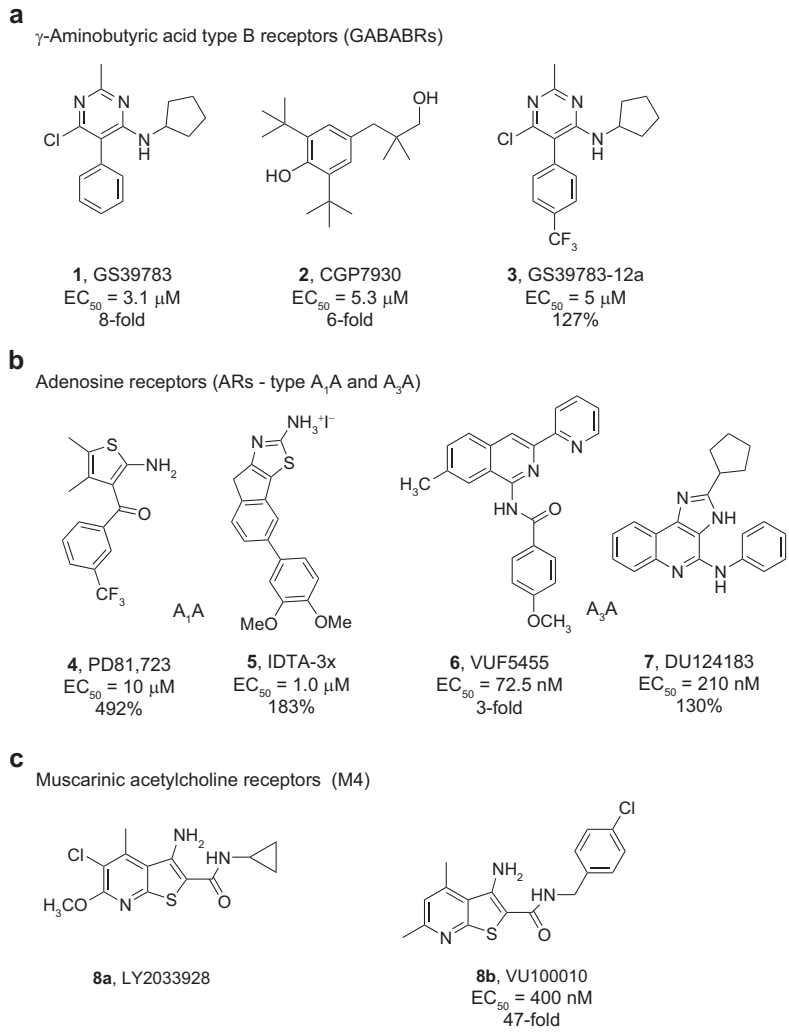

Figure 4. Representative PAMs of Family A GPCRs.

Orthosteric *versus* Allosteric Ligands. All GPCRs possess a distinctive binding site for their respective endogenous ligand(s) that is known as the orthosteric site. Ligands that bind to this site are considered classical or traditional orthosteric ligands. This group includes small-molecule agonists, partial agonists, antagonists, and inverse agonists; in general, the most physiologically common and relevant of these ligands are the endogenous agonists.

The location and mechanism of agonist-induced activation differ considerably from one class of receptors to another; however, common themes have emerged. For all GPCRs, binding and activation by an orthosteric agonist ultimately results in a structural rearrangement of the receptor that results in increased affinity for G-proteins. In the case of most biogenic amines, nucleosides, and lipid moieties, ligand binding occurs within the hydrophobic core of the receptor. By contrast, small-peptide hormones bind to the core, extracellular loops,

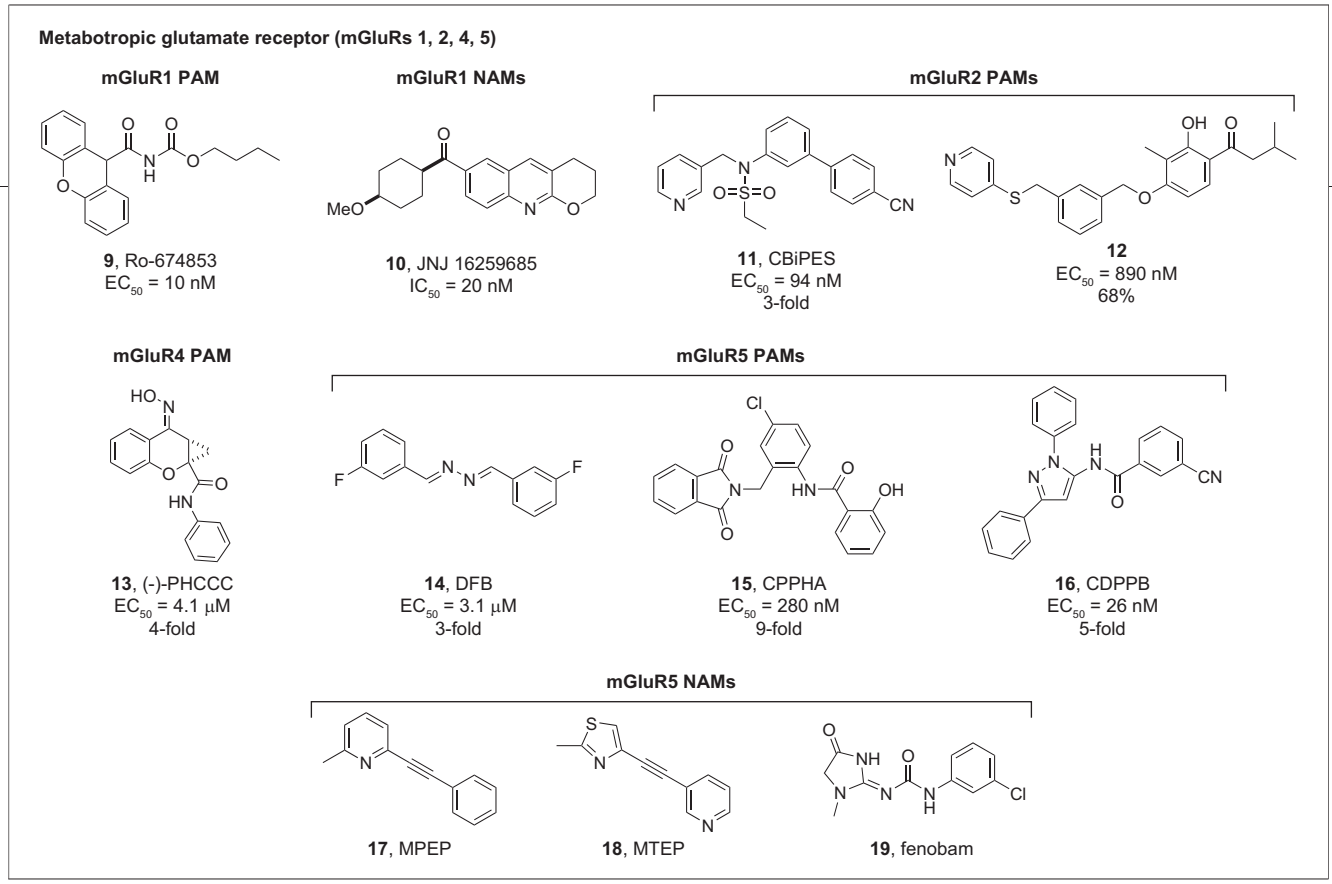

Figure 5. Representative PAMs and NAMs of Family C GPCRs.

and the N-terminal segment of their respective GPCR. Larger proteins, and glycoproteins in particular, generally bind to the N-terminal tail, which then moves down to establish ligand-loop interactions to activate the receptor. Similarly, many neurotransmitters including GABA and glutamate bind to the large N-terminal tails found on metabotropic class C neurotransmitter receptors, causing a conformational change that brings the N-terminus down to the transmembrane domain to induce activation. The precise structural changes involved in transducing ligand-binding to G-protein-binding are diverse and complex, but they can be generalized to involve the disturbance of ionic interactions between transmembrane helices three and six (*15, 16*). This facilitates binding of the G-protein predominately by helices two and three as well as the C-terminal tail (*7*).

In the case of drug discovery, much of the field's history has revolved around the identification and study of small molecules that act as orthosteric ligands at a given target receptor to elicit a pharmacological effect. These compounds compete with the endogenous ligand(s) and thus preclude simultaneous occupation of the receptor by both molecules. Figure 2 depicts the orthosteric ligand binding site for each of the three main GPCR classes along with representative endogenous (orthosteric) agonists for each class. Classical drug discovery focused on radioligand binding assays to identify "hits", which were, by default, orthosteric ligands.

In addition to orthosteric sites, many GPCRs have been found to possess allosteric (Greek, "other site") binding sites that are spatially and often functionally distinct (*17–19*). The presence of allosteric sites allows for numerous additional ligand−receptor interactions beyond those associated with the orthosteric site. Allosteric agonists, antagonists, and inverse agonists for a given GPCR will bind to the allosteric site and induce a similar effect as their orthosteric relatives. Beyond such types of ligands, allosteric modulators bind to an allosteric site where they stabilize a receptor conformation and equilibrium shift that increases or decreases the affinity and/or efficacy of an orthosteric agonist at the receptor, without activating the receptor on its own (*17–19*). The modulator lacks intrinsic agonist or inverse agonist activity, and thus increased or decreased signaling *via* the receptor occurs *only* in the presence of an orthosteric agonist. Such ligands are often respectively termed positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), and examples of each are highlighted in Figure 4 and Figure 5 for Families A and C, respectively (*17–19*). With the evolution of high-throughput screening (HTS) and functional assays, scientists were able to identify molecules that af-

fect the function of GPCRs irrespective of binding mode. Figure 6 highlights how a functional HTS screen, typically Ca^{2+}-mobilization measured by fluorescence with a calcium-sensitive dye, is designed to identify positive allosteric modulators (*20*).

The sometimes confusing concept of allosteric modulation by a potentiator is often best visualized by looking at the effect of a PAM in two simple cell-based pharmacological assays: a full concentration response curve (CRC) of an orthosteric agonist in the presence of increasing concentrations of potentiator (Figure 7, panel a) and the full CRC of a potentiator in the presence of a fixed low concentration of orthosteric agonist (Figure 7, panel b). Together, these assays demonstrate a shifting of the orthosteric agonist potency (and/or efficacy, depending on the potentiator), which can be translated into a quantitative "fold over baseline" or "fold-shift" as a measure of potentiation. Although it is not yet well understood what degree of fold-shift is required for significant effects *in vivo*, some published examples demonstrate *in vivo* significance with as little as 3−6-fold leftward shift of the orthosteric agonist CRC (*21*). As one would expect, the specific physiological or pathological context is likely to determine the degree of potentiation required to observe *in vivo* effects, which cannot be easily generalized. Furthermore, the exact mechanism(s) involved in mediating the effects of a potentiator are likewise in need of further study.

The identification of small-molecule ligands that have both allosteric agonist and potentiator activity, termed ago-allosteric modulators, has furthered the number of possible receptor−ligand interactions. Such compounds bind to allosteric sites to exert potentiation effects but can also act as agonists in the absence of orthosteric ligand. This agonist effect is often seen at higher concentrations, with the effect transforming into a pure potentiation at decreasing concentrations (Figure 8). Clearly, these receptor−ligand-signaling phenomena open the door to a host of speculation about the specific receptor conformations and binding dynamics associated with ago-allosteric modulators, and recent publications address these issues in more depth (*22, 23*).

In addition to PAMs and NAMs, allosteric binding sites on GPCRs allow other novel modes of receptor modulation. Allosteric ligands can display a phenomenon referred to as neutral cooperativity, also coined pharmacological silence. These ligands do not activate

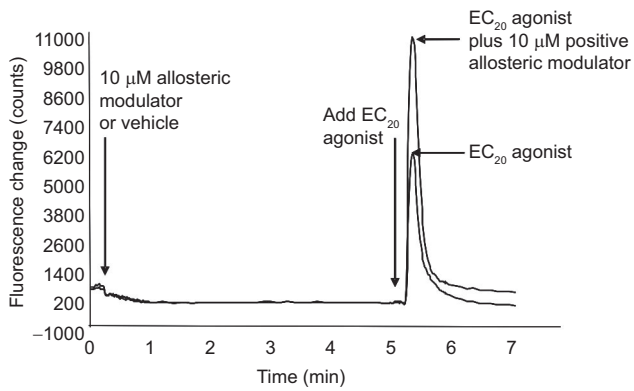

Figure 6. HTS screen for a positive allosteric modulator employing calcium fluorescence. To detect potentiation in the screening assay, test compounds were added after 10 s of baseline determination. Five minutes later, a fixed EC_{20} concentration of orthosteric agonist was added. An "active" was identified as a compound that caused no response in the absence of the orthosteric agonist but a significant (≥2-fold) increase in the response to the EC_{20} concentration of orthosteric agonist.

or inactivate the GPCR in the presence or absence of orthosteric agonist but block the activity of both PAMs ands NAMs by occupying the allosteric site. The first example of this was described for allosteric ligands of mGluR5 within the DFB (**14**) series (Figure 5) (*24*). Conn et al. (*25*) recently reported on the discovery of a fundamentally new mode of GPCR modulation with the report of mGluR5 allosteric "partial antagonists". Partial antagonists fully occupy the MPEP (**17**) binding site on the mGluR5 receptor but only partially block agonist response, resulting in partial mGluR5 inhibition (*25*). As reported by Rodriguez and coworkers (*23*), this effort identified three compounds that only partially inhibited or had no functional effects on mGluR5 response. Coined M-5MPEP (**20**) and Br-5MPEPy (**21**), these compounds represented the first partial antagonists of mGluR5 inducing a maximal mGluR5 inhibition of ~50%, along with 5MPEP (**22**), another neutral, pharmacologically silent allosteric site ligand (Figure 9) (*25*).

KEYWORDS

Ago-allosteric modulator: An allosteric ligand that functions as both an allosteric modulator and as an agonist on its own (though the latter is usually only at higher concentrations.

Allosteric agonist: A ligand that is capable of receptor activation on its own by binding to a recognition site that is distinct from the orthosteric site.

Allosteric modulator: A ligand that increases or decreases the action of an orthosteric ligand (agonist or antagonist) by binding at an allosteric site. The modulator may enhance the affinity and/or efficacy of the orthosteric ligand while exerting no effects on its own.

Allosteric site: A ligand binding site that is distinct from the orthosteric binding site. In truest form, there should be no overlap with the orthosteric binding site.

Orthosteric site: The binding site of the endogenous agonist.

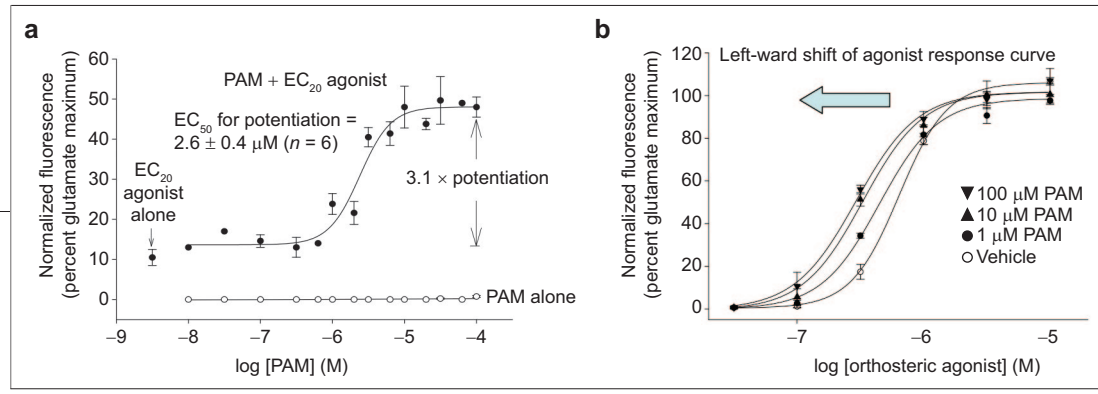

Figure 7. Effect of a PAM in two simple cell-based pharmacological assays. a) A small-molecule PAM potentiates GPCR activation by orthosteric agonist. A range of concentrations of a small-molecule PAM were added to cells after 10 s of baseline determination. Five minutes later, a fixed concentration (~EC_{20} concentration) of agonist (glutamate in this figure) was added, and the Ca^{2+} response was measured. b) A full CRC of the same small-molecule PAM in the presence of a fixed, low concentration of orthosteric agonist. The small-molecule potentiation of response is manifested as increased agonist sensitivity, that is, the EC_{50} of the orthosteric agonist is leftward shifted.

Sharma and co-workers (*26*) recently reported on the SAR of another mGluR5 partial antagonist, **23**, identified in a high-throughput screening campaign. The analogs of HTS partial antagonist lead **23** within a small library elucidated a "molecular switch" to modulate pharmacological activity (Figure 10). Lead **23**, with an unsubstituted distal phenyl ring, fully occupied the MPEP binding site, possessed an IC_{50} of 486 nM, but only afforded partial response (29% response, 71% partial antagonism), that is, allosteric partial antagonism. Incorporation of small chemical moieties in the 3-position of the distal phenyl ring, such as a 3-methyl group, delivered **24**, a full noncompetitive mGluR5 antagonist (IC_{50} = 7.5 nM). When the methyl group is moved from the 3-position to the 4-position as in **25**, an efficacious (99% of glutamate max) mGluR5 positive allosteric modulator resulted (EC_{50} = 3.3 μM, 4.2-fold shift). The observation of a conserved molecular switch, accessed by toggling between 3- and 4-substitution on the distal phenyl ring, within this chemical series is unprecedented and once again highlights the complexities involved in the optimization and development of allosteric ligands.

The discovery of allosteric modulators and the intricate mechanisms underlying their pharmacological properties clearly demanded revision and expansion of classical receptor models. However, even prior to consideration of allosteric modulators, the early two-state receptor models had evolved concurrently with the progressive elucidation of more complex ligand−receptor interactions (*18*, *27*). First, the ternary complex model (TCM) arose as an expansion of the simple linear two-state model (Figure 11, panel a), taking into account not only the interaction between a ligand and its receptor but also the active receptor (R*) and G-protein and giving rise to a four-point 2D model (Figure 11, panel b). The extended TCM was then introduced to include the spontaneous activation of a receptor that can interact with a G-protein even in the absence of agonist binding (*i.e.*, agonist-independent spontaneous signaling), producing a six-point 2D model (Figure 11, panel c). Beyond this, the eight-point 3D cubic TCM encompasses the

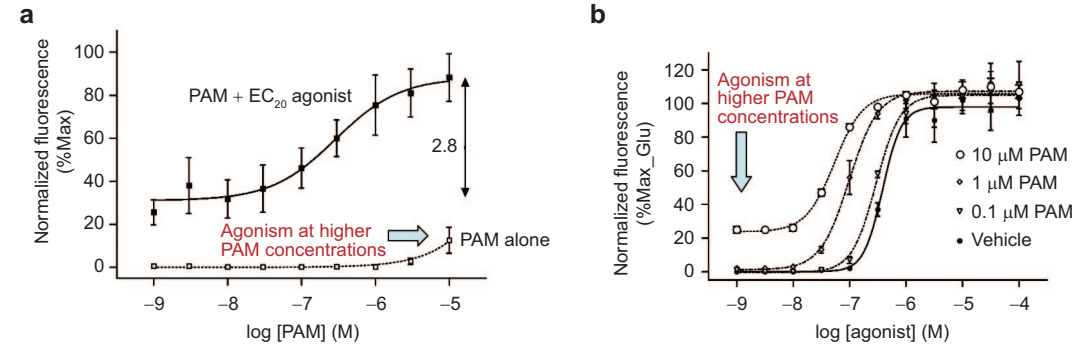

Figure 8. Concentration response curves for a prototypical ago-allosteric modulator. a) At low compound concentrations, the molecule behaves like a positive allosteric modulator, eliciting no activation of the receptor in the absence of an EC_{20} of the orthosteric agonist. Only at higher concentrations is agonism observed. b) In the present case, at concentrations <10 μM, this small-molecule PAM displays ~25% agonism. Interestingly, despite the agonist activity, this molecule retains complete GPCR subtype selectivity; thus, a more accurate descriptor would be that the molecule possesses a degree of allosteric agonism.

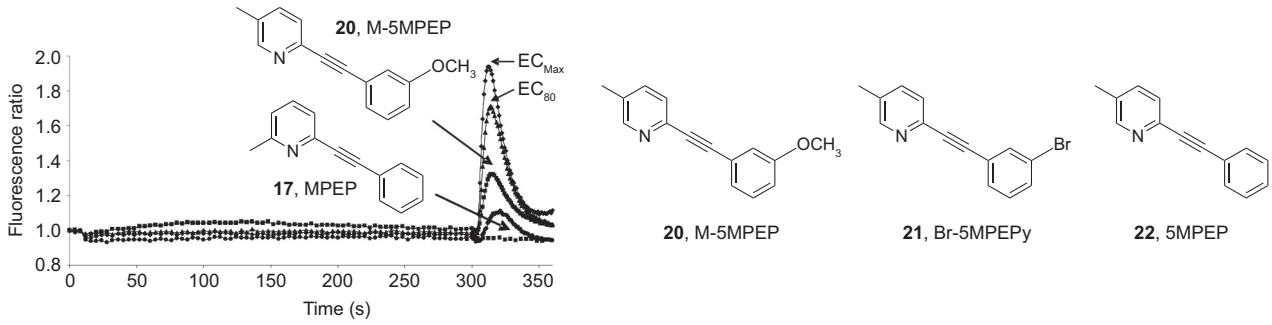

Figure 9. MPEP (17, 10 μM) blocks calcium fluorescence response to glutamate, whereas saturating concentrations of representative mGluR5 partial antagonists, such as 20, only partially inhibit calcium fluorescence response to glutamate.

thermodynamically required (but most likely physiologically rare) consideration of an interaction between the receptor in the inactive (R) state and the G-protein that does not cause signaling (Figure 11, panel d) (*28*). From here, the introduction of allosteric modulators to the system has given rise to yet more complex models, such as the 16-point quaternary complex model of allosteric GPCR interactions (not shown, see ref 18). This model robustly considers all thermodynamically possible interactions between a receptor and an orthosteric ligand, an allosteric ligand, and a G-protein. It is important to note that all such models are strictly speaking still two-state models in that the receptor is conceived of as binary (either R or R* state); however, there are proposed *n*-state models as well (*29*).

Ultimately, a simple and intuitively accessible model can be used to describe allosteric modulation of GPCRs for those seeking a more pragmatic solution. The original TCM used to describe ligand, receptor, and G-protein interactions can be modified to describe interactions between two ligands (*e.g.*, an orthosteric agonist and an allosteric modulator) on one receptor. This model (Figure 11, panel e), often referred to as the allosteric TCM (ATCM), uses equilibrium dissociation constants for the interactions between the receptor and each ligand (K_a for ligand A; K_b for ligand B), as well as a cooperativity factor (α) that denotes the mutual effect of the two ligands on each other's affinity for the receptor (*18*). An $\alpha < 1.0$ refers to positive cooperativity, an $\alpha > 1.0$ refers to negative cooperativity, and an $\alpha = 1.0$ means that binding of either ligand to the receptor does not alter the affinity of the other ligand for the receptor (*i.e.*, a neutral modulator). Further, the β parameter can be added as a subtle but highly useful extension to the ATCM in order to include effects of an allosteric modulator on the efficacy (as distinct from the affinity) of another ligand that binds the receptor, such as the orthosteric agonist. Interestingly, some ligands can reduce the efficacy but increase the affinity of the orthosteric agonist for the receptor. Although a further and more detailed examination of receptor theory and allosteric modulator activity modeling is clearly beyond the scope of this Review, readers are referred to a number of previous publications on this topic (*6, 18, 19, 22, 27, 40*).

Significance of Allosteric GPCR Modulation. The discovery of GPCR allosterism and the identification of small-molecule allosteric ligands have had a wide range of implications across both the basic sciences and the

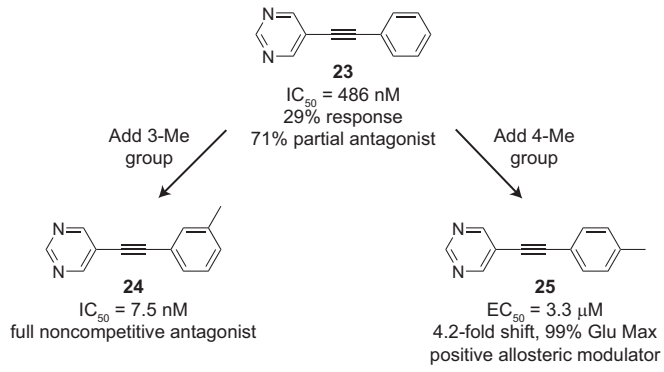

Figure 10. SAR of an mGluR5 partial antagonist and identification of a "molecular switch". Compound 23 is an mGluR5 partial antagonist (IC_{50} = 486 nM, 71% antagonism). Incorporation of a 3-methyl group in the distal phenyl ring affords 24, a full noncompetitive antagonist with increased activity (IC_{50} = 7.5 nM). Introduction of a 4-methyl group in the distal ring provides 25, an mGluR5 positive allosteric modulator (EC_{50} = 3.3 μM, 4.2-fold shift).

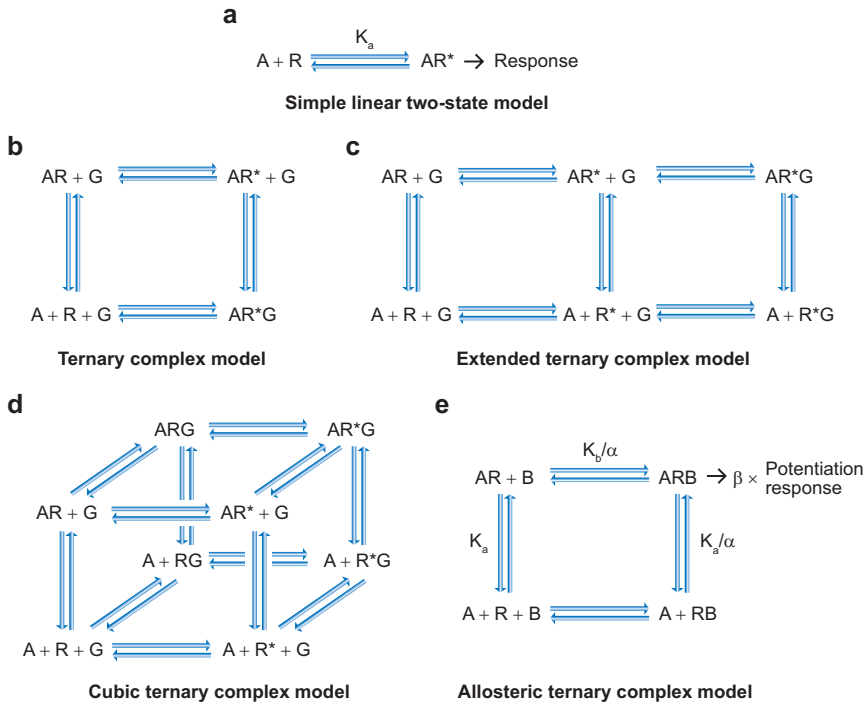

Figure 11. Models of GPCR ligand–receptor interactions. a) A simple linear two-state model employing a single dissociation constant governed by the law of mass action. b) The initial ternary complex model for ligand, receptor, and G-protein interactions. c) The extended ternary complex model, which includes the scenario of constitutive or agonist-independent receptor activation. d) The cubic ternary complex model, which adds the possibility of active receptor and G-protein association that does not causing signaling. e) The allosteric ternary complex model, a concise framework for modeling the interaction of two ligands (*e.g.*, an orthosteric agonist and an allosteric modulator) on a receptor, taking into account both cooperativity between the ligands (α) and the effect of one ligand on the other's efficacy (β).

drug discovery field. One of the main reasons for this is that allosteric binding sites can allow for more targeted compounds because of the increased potential for receptor subtype selectivity (*18*). This is often due to the highly conserved amino acid sequences coding for the orthosteric binding site across all receptor subtypes, precluding discovery of highly subtype-selective compounds; however, allosteric sites may be less conserved across subtypes, providing a means for true selectivity. For example, despite much effort by medicinal chemists, traditional orthosteric agonists of the muscarinic acetylcholine receptor aimed at targeting one of the five subtypes (M1–M5) have shown poor selectivity, but an allosteric ligand, such as the M4 PAM **8** (Figure 4), and allosteric (ectopic) M1 agonists AC-42 (**26**), *N*-desmethyl clozapine (**27**), and TBPB (**28**) display unprecedented subtype selectivity (Figure 12) (*30–33*).

Such increased selectivity found with allosteric ligands has the potential to translate into very exciting and clearly needed progress in numerous areas of pharmacology and drug discovery. Problems associated with lack of subtype selectivity have precluded market approval for a number of drug candidates because of the dose-limiting side effects of traditional agonists. For example, a number of muscarinic agonists evaluated in clinical trials for the treatment of Alzheimer's disease, including xanomeline, milameline, sabcomeline, cevimeline, and talsaclidine, have all shown therapeutic efficacy but ultimately failed because of poor subtype selectivity and associated side effects (*34*). In the case

of small-molecule compounds for use as research tools, lack of selectivity has forced researchers to rely largely on genetic approaches such as receptor subtype knockout mice. Generation of these mice is time- and labor-intensive, and they can suffer from lack of relevance due to emergence of compensatory mechanisms during development that distort the effect of the knockout. Thus, allosteric modulators can potentially facilitate large strides forward in basic and applied pharmacological science.

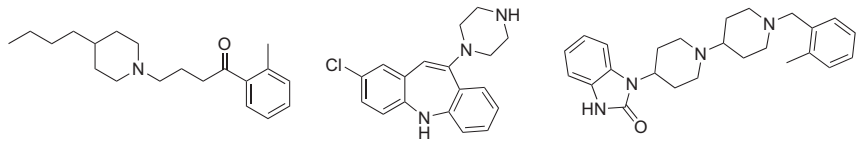

Figure 12. Structures of M1 allosteric agonists AC-42 (26), *N*-desmethylclozapine (27), and TBPB (28). By virtue of activating the M1 receptor at an evolutionary nonconserved allosteric site, these ligands display unprecedented mAChR subtype selectivity, only activating M1. Note that these allosteric ligands bear no similarity to the orthosteric agonist acetylcholine.

An advantage that allosteric potentiators in particular can provide over traditional orthosteric agonists is the presence of an effect ceiling (*18*). Because of the dependence upon the endogenous orthosteric agonist for signaling, the presence of even an extremely high concentration of potentiator (as in the case of an overdose) will not translate into increased receptor activation beyond a certain point or ceiling. This advantage is best exemplified with the well-known benzodiazepine drugs, which are $GABA_A$ receptor modulators. Although this GABA receptor subtype is a ligand-gated ion channel and not a GPCR, this case demonstrates that the ceiling effect can confer overdose safety, as benzodiazepine overdoses are usually not fatal despite the highly therapeutic effects of these drugs for anxiety and sleep disorders (*35*). Whether this is a common theme for all allosteric potentiators, including those of GPCRs, remains to be seen; however, if a given GPCR potentiator does not significantly boost the endogenous orthosteric agonist efficacy but only its potency, it is likely that benefits of the effect ceiling could confer *in vivo* drug safety.

Yet another advantage that some allosteric GPCR modulators possess over traditional orthosteric agonists is that their modulation of receptor signaling remains physiologically relevant (*18*). This could be especially important in the case of neurotransmitter receptor targets, where patterns and oscillations of neuron firing and synaptic neurotransmission occur in extremely complex circuits and networks to mediate sophisticated neurological functions, including those underlying cognition, attention, language, and more. Chronic activation of such receptors by traditional agonists robs the system of its precise regulation, and this may translate into increased side effects and/or lack of efficacy. In contrast, an allosteric potentiator will preserve the physiological relevance of receptor signaling and neurotransmission while at the same time boosting the efficiency of the endogenous neurotransmitter. 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 (*36*). Furthermore, lack of chronic receptor activation may cause less receptor desensitization or internalization over time, which could allow for a potentiator to overcome the problem of diminishing therapeutic efficacy that is seen with many chronically administered orthosteric agonists.

Two allosteric modulators of GPCRs have entered the market, further exciting the field about the prospect of this new mode of GPCR modulation. The first to enter the market was Cinacalcet (**29**), a positive allosteric modulator of the calcium sensing receptor (CaSR) (*37*). The CaSR is involved in regulation of calcium homeostasis and functions in renal calcium resorption, as well as in maintenance of intracellular inositol triphosphate levels (*37*). Cinacalcet was found to have an EC_{50} of 34 nM and to maximally potentiate the endogenous agonist activity by 2-fold (Figure 13) (*38*). For disorders where a CaSR-related deficiency occurs, drugs like these can

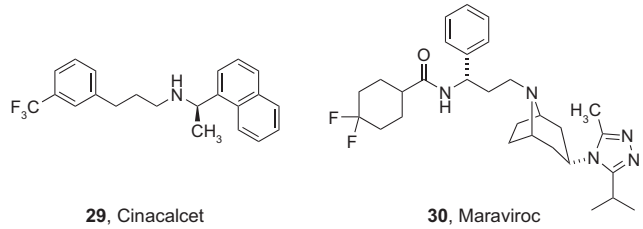

Figure 13. Structures of the two marketed GPCR allosteric modulators: Cinacalcet (29), a PAM of the calcium sensing receptor, and Maraviroc (30), a NAM of CCR5.

play an integral role in the therapy. Shortly thereafter, the noncompetitive CCR5 antagonist (NAM) Maraviroc (**30**) was launched for the treatment of HIV, by blocking the interaction between HIV and the chemokine receptor CCR5 on host cells (*39*).

Conclusion and Summary. GPCRs are experiencing a renaissance, both in terms of basic pharmacological understanding and therapeutic potential. Already the largest single class of marketed therapeutic agents (as agonists, antagonists, and inverse agonists), GPCRs, through new allosteric modes of target modulation (PAMs, NAMs, allosteric agonism, partial antagonism), could potentially capture an even larger market share because of the major advantages of allosteric modulation (subtype selectivity, saturable effects, and mimicking of physiological responses). Cinacalcet (a PAM) and Maraviroc (a NAM) demonstrate that allosteric modulation of GPCRs is a safe and therapeutic relevant approach for GPCR activation and inactivation, respectively. Unlike the 50-plus years of orthosteric site GPCR modulation, exploiting allosteric sites for GPCR modulation is in its infancy and holds great promise for basic discovery and clinical translation of GPCR targets previously considered intractable.

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