Allosteric Modulation of G Protein–Coupled Receptors

Lauren T. May,[1] Katie Leach,[2]
Patrick M. Sexton,[2] and Arthur Christopoulos[2]

[1]Department of Pharmacology, University of Melbourne, 3010, Parkville, Victoria, and [2]Drug Discovery Biology Laboratory, Department of Pharmacology, Monash University, Clayton, 3800, Australia; email: arthur.christopoulos@med.monash.edu.au

Annu. Rev. Pharmacol. Toxicol. 2007. 47:1–51

First published online as a Review in Advance on September 29, 2006

The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org

This article's doi:
10.1146/annurev.pharmtox.47.120505.105159

Copyright © 2007 by Annual Reviews.
All rights reserved

0362-1642/07/0210-0001$20.00

Key Words

drug discovery, efficacy, receptor theory, structural biology

Abstract

The past decade has witnessed a significant growth in the identification of allosteric modulators of G protein–coupled receptors (GPCRs), i.e., ligands that interact with binding sites that are topographically distinct from the orthosteric site recognized by the receptor's endogenous agonist. Because of their ability to modulate receptor conformations in the presence of orthosteric ligand, allosteric modulators can "fine-tune" classical pharmacological responses. This is advantageous in terms of a potential for engendering greater GPCR subtype-selectivity, but represents a significant challenge for detecting and validating allosteric behaviors. Although allosteric sites need not have evolved to accommodate endogenous ligands, there are a number of examples of where such modulators have been shown to contribute to physiological or pathophysiological processes. Studies are also beginning to unravel the structural basis of allosteric modulation of GPCRs. It remains to be determined whether such modulation represents interactions within monomers versus across dimers.

INTRODUCTION

Allosteric site: a binding site on the receptor that is topographically distinct from (does not exhibit any overlap with) the orthosteric site

Cooperative binding: the binding of more than one molecule of the same ligand to a receptor complex. This term is often also used more loosely to describe the binding of two or more molecules of any type binding to the same receptor

G protein–coupled receptors (GPCRs) constitute the largest family of integral membrane proteins, accounting for approximately 3%–4% of the human genome (1). Perhaps more than any other type of protein, GPCRs have evolved to recognize a plethora of endogenous stimuli, and act to transmit messages encoded in stimuli from the exterior to the interior of the cell. The ubiquitous cell surface distribution of these proteins and their involvement in virtually all biological processes accounts for the fact that the largest proportion of currently marketed medicines target GPCRs (2).

Despite the eminent tractability of GPCRs as drug targets, significant challenges remain with respect to understanding mechanisms of drug action at GPCRs and translating this understanding into more selective, effective medicines. Some of these challenges relate to a relative lack of appreciation of the paradigms of protein activity that have potentially enormous implications for drug discovery at GPCRs. For example, most proteins contribute to signaling networks that involve protein-protein interactions, yet the notion that GPCRs may form dimers or higher order oligomers, or that they partake in accessory protein interactions beyond the well-established G protein interaction, has only gained prominence within the past decade (3, 4). Similarly, and despite evidence of the phenomenon peppering the literature since the late 1960s, the past decade has also witnessed the development of interest in targeting ligands to allosteric sites on GPCRs, i.e., domains that are topographically distinct from that utilized by endogenous agonists. Much of this interest has been driven by the pharmaceutical industry and the change in high-throughput screening methods from predominantly binding-based to functional. However, the relevance of GPCR allosterism is not restricted to drug discovery but has important implications for structure-function analyses of GPCR action.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

CONCEPTS AND TERMINOLOGY

The phenomenon of allostery has long been observed in the study of biological processes, well before the term allosteric was actually coined. This term has since come to encompass different, albeit related, mechanisms by which protein function can be regulated and fine-tuned in either a positive or negative direction. Two broad concepts underpin the majority of studies on allosterism. The first, developed initially in the field of enzymology, is that many proteins possess more than one binding site. For example, early studies of Bohr on hemoglobin (perhaps the most famous experimental model of allosterism) revealed that the protein could simultaneously bind more than one molecule of oxygen (5), a phenomenon termed cooperativity. A.V. Hill derived his famous equation, often used to empirically fit dose-response curve data, as an attempt to quantify cooperative binding reactions (6). Experiments with ligand-gated ion channels, such as GABA_A and nicotinic acetylcholine receptors (nAchR), also helped disseminate the notion that cooperative binding is likely a general phenomenon for the function of many proteins. Importantly, the binding of successive equivalents of ligand to a cooperative protein is often associated with an alteration in the affinity of the remaining (free) binding sites for subsequent ligand, highlighting the fact that the sites are conformationally linked.

Allosteric modulator: any ligand that binds to an allosteric site

The second important concept associated with allosterism was best enunciated in the studies of Wyman (7), who highlighted the ability of proteins to undergo global conformational changes that yield binding pockets with different affinities for ligands. It follows that the binding of a given ligand to such proteins can bias the conformational states toward those with the highest affinity for that ligand. Because protein conformations govern their behavior, the consequences of this important insight cannot be overstated. For example, early studies by Katz (8) on the nAchR explicitly incorporated receptor isomerization between different states, separating the binding step from the activation (i.e., gating) and desensitization steps. The same concept also predicted the phenomena, now experimentally well established, of constitutive activity and inverse agonism; thermodynamic considerations require that isomerization of proteins between multiple conformational states must include active states (no matter how transiently) even in the absence of bound ligands (6).

The concepts of site-site interactions and global protein conformational changes coalesced in a series of seminal papers by Monod and colleagues, where the word allosteric was first coined. The authors began by tackling the phenomenon of end-product inhibition in enzymes, noting that many inhibitors were often strikingly diverse in structure from the enzyme's substrate. This led to the suggestion that the inhibitors utilized a different binding site than the substrate, but that the sites were conformationally linked such that the inhibition could be transmitted from the inhibitor site to the substrate site (9). This culminated in a proposal of a formal mechanism for allosteric proteins, which required an oligomeric architecture, cooperativity in binding, and global conformational changes between states that were preferentially selected by different ligands (10). This last, strict definition of allosteric proteins is at odds with the more widespread use of the term today to describe multi-site interactions on proteins, irrespective of whether they are oligomeric or not, and/or the transition of proteins between conformational states, irrespective of whether they possess multiple binding sites or not. As such, discussions of allosterism require a frame of reference and clear definitions of what is meant when using the term allosteric.

When dealing with GPCRs, there is a third concept that is of relevance to allosteric regulation, namely, that GPCRs must translocate within the membrane and interact with other proteins to transmit external stimuli to the cell and alter a response. The sites of interaction of the GPCR agonist and that of accessory interacting cellular proteins are different and topographically distinct. Thus, GPCRs are naturally allosteric in that they possess more than one type of binding site, with the G protein itself being the best-known allosteric modulator of agonist binding to GPCRs (6, 11). Allosteric protein-protein interactions are highly likely to occur for GPCRs because these receptors interact with a variety of cellular proteins, in addition to or independently of the G proteins (4). However, such interactions are beyond the scope of the current review. Rather, we focus on GPCRs as pharmaceutical targets for exogenous allosteric modulators, although some discussion is reserved for putative endogenous modulator ligands.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

Allosteric interaction: an interaction between ligands that bind to distinct, nonoverlapping, but conformationally linked, recognition sites on the receptor macromolecule

Orthosteric site: the primary binding site on the receptor that is recognized by the endogenous agonist for that receptor

Allosteric agonist (or activator): allosteric modulators that mediate receptor activation in their own right by binding to a recognition domain on the receptor macromolecule that is distinct from the orthosteric site

The necessity for a distinction between the site on a GPCR recognized by its endogenous agonist and other topographically distinct binding sites has led to the term orthosteric being used to refer to the endogenous agonist binding site (12). An allosteric interaction occurs between the orthosteric site and any additional conformationally linked site when both sites are occupied by ligands. Allosteric modulators are thus defined as ligands that bind to an allosteric site on the GPCR to modulate the binding and/or signaling properties of the orthosteric site. This is a more restricted definition than the one in Wyman's model of interchanging conformational states in which an allosteric modulator is any ligand that preferentially selects one conformational state over another, irrespective of where it binds. Clearly, this latter definition is applicable to all protein-binding ligands!

There are different "flavors" of allosteric modulators, as defined recently by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (12), although allosteric modulators have the potential to display a combination of these properties (see Mechanisms Underlying Allosteric Modulation, below). For example, because allosteric and orthosteric sites are conformationally linked, allosteric agonists also have the capacity to modulate binding and/or signaling of orthosteric ligands; the extent to which such modulation occurs over and above agonistic properties of the allosteric ligand will depend on the nature of the orthosteric ligand and the experimental conditions. Moreover, the same allosteric modulator can act at the same receptor and be an enhancer of one orthosteric ligand, an inhibitor of another, and exert no effect on a third. These varying responses to allosteric modulators render them potential treasure-troves of pharmacological tools for sculpting biological responses but also make the task of detecting and exploiting such modulators more difficult than the study of orthosteric agonists and antagonists. **Table 1** lists examples of putative allosteric modulators at all three major subclasses of mammalian nonsensory GPCRs.

MECHANISMS UNDERLYING ALLOSTERIC MODULATION

It is now apparent that GPCRs have a far wider range of behaviors than previously appreciated (e.g., ligand binding, G protein coupling, second messenger generation, ion channel coupling and gating, accessory protein interaction, oligomerization, phosphorylation, internalization, etc.), each potentially associated with a unique spectrum of conformational states. Thus, although classic paradigms of GPCR activity assume that all functional outcomes following the initial binding event are sequentially linked, ideas based on varying receptor conformations ("conformational hypothesis") make it clear that this need not be the case, and that different ligands can differentially bias GPCR conformations toward one type of behavior versus another (13). Allosteric modulators of GPCRs add another layer of complexity because they engender additional conformational states both on their own and in the presence of a bound orthosteric ligand. As a consequence, allosteric ligands have the potential to contribute significant "texture" to the pharmacology of both traditional (orthosteric) agonists and antagonists in a ligand-, receptor-, and even cell-dependent manner. The great challenges for studies on GPCR allosterism, therefore, relate to the need

Table 1 Selected examples of putative allosteric modulators of GPCRs

Receptor	Example modulator(s)
Family A	
Adenosine A_1	PD 81723, LUF 5484
Adenosine A_{2A}	Amilorides
Adenosine A_3	VU5455, VU8504, DU124183
Adrenoceptor α_1	Amilorides, benzodiazepines, conopeptide ρ-TIA
Adrenoceptor $\alpha_{2A,}$ α_{2B}	Amilorides
Adrenoceptor α_{2D}	Agmatine
Adrenoceptor β_2	Zinc
Cannabinoid CB_1	Org 27569, Org 27759, Org 29647
Chemokine CXCR1, CXCR2	Repertaxin
Chemokine CXCR3	IP-10, I-TAC
Chemokine CCR5	Trichosanthin, AK602, AK530, TAK779, SCH 351125, 873140
Chemokine CXCR4	RSVM, ASLW, Trichosanthin
Chemokine CCR1, CCR3	UCB35625
Dopamine D_1	Zinc
Dopamine D_2	Amilorides, zinc, L-prolyl-L-leucylglycinamide
Endothelin ET_A	Aspirin, sodium salicylate
Growth hormone secretagogue receptors	L-692429, GH-releasing peptide 6
Muscarinic M_1–M_5	Gallamine, alcuronium, brucine, W84, C_7/3-phth, WIN 62577, AC-42, thiochrome, MT7, MT3, strychnine, staurosporine, tacrine, McN-A-343
Neurokinin NK_1	Heparin
Opioid μ, δ	Cannabidiol
Purine $P2_{Y1}$	2,2′-pyridylsatogen tosylate
Serotonin $5HT_{1B/1D}$	5HT moduline
Serotonin $5HT_{2A}$, $5HT_7$	Oleamide
Serotonin $5HT_{2C}$	Oleamide, PNU-69176E
Family B	
CRF1 Receptor	Antalarmin, NBI 35965, DMP696, NBI 27914, BIBN4096BS
Glucagon	Bay27–9955, L-168049
GLP1 receptor	T-0632
Family C	
Calcium sensing receptor	Fendeline, cinacalcet, NPS 467, NPS 568, L-amino acids, NPS 2143, Calhex 231
$GABA_B$	CGP7930, CGP13501, GS39783
Glutamate $mGluR_1$	(-)-CPCCOEt, Ro 67–7476, Ro 01–6128, BAY36-7620, [^{3}H]R214127, NPS 2390, EM-TBPC, *cis*-64a
Glutamate $mGluR_2$	LY 487379, biphenyl-indanone A, LY-181837, LY-354740
Glutamate $mGluR_3$	LY-354740
Glutamate $mGluR_4$	SIB-1893, MPEP, (-)- PHCCC
Glutamate $mGluR_5$	MPEP, DFB, DmeoB, DCB, CPPHA, CDPPB
Glutamate $mGluR_7$	AMN082

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

to appreciate the diversity of possible allosteric effects on GPCR biology and the ability to delineate the molecular processes governing protein allostery.

In recent years, new methodologies have been developed for studying allosterism at the molecular level. For example, a novel computational procedure, termed statistical coupling analysis (SCA), mines large databases of homologous proteins and compares their sequences in order to identify evolutionarily covarying residues (14). Given its reliance on the availability of large databases, it is unlikely that SCA will identify novel allosteric networks between orthosteric and small-molecule allosteric sites specific to individual GPCRs. Nevertheless, it represents a conceptually elegant approach for building a picture of allosteric networks in the absence of tertiary structure information. Additional computational methods to delineate allosteric networks within proteins include the nonequilibrium molecular dynamic simulation procedure of anisotropic thermal diffusion (which monitors the propagation of thermal energy throughout a protein from a local site), evolutionary trace analysis, and normal mode/principal component analyses (15, 16). NMR hydrogen/deuterium exchange techniques are experimental methods key for mapping fluctuations in protein structure derived from distinct conformational states (17).

The significant experimental and computational advances being made in the study of protein conformational states have the potential to describe the molecular nature of allosterism. However, many of these methods are technically challenging, have not been applied to most proteins, and may not be readily amenable to the drug discovery process involving allosteric modulators of GPCRs. Perhaps a currently more tractable problem is the other challenge facing drug discovery at GPCRs, namely, a conceptual understanding of the manifestations of allosterism at these receptors. To do so, it is necessary to balance two potentially contrasting goals: quantitative models that describe experimentally-observed allosteric behaviors and parsimonious mechanisms that can be applied to data generated from GPCR discovery programs. To this end, the most common approaches currently utilize macroscopic mass action schemes that encapsulate the allosteric interaction in a handful of thermodynamic parameters, the latter of which describe the allosteric interaction in terms of effects on binding affinity and/or effects on efficacy.

Allosteric Effects on Affinity

A receptor that binds an allosteric ligand represents a new structure owing to the conformational change that ensues. Accordingly, the rates at which an orthosteric ligand associates or dissociates from its binding site on this receptor conformation need not be the same as rates for the unoccupied receptor. Because ligand affinity is defined as the ratio of ligand association to dissociation rates, a key manifestation of an allosteric interaction is an alteration in the affinity of an orthosteric ligand for its binding site. Hence, allosteric enhancement can arise from an increase in ligand association rate and/or a decrease in ligand dissociation rate (the latter being more common), whereas allosteric inhibition can arise from opposite changes. Both association and dissociation rates can change in the same direction, but enhancement or inhibition can still result depending on the relative effect on one parameter compared with the other.

The simplest model of allosteric interactions, which also forms the basis of many quantitative studies of GPCR allosterism, is the allosteric ternary complex model (ATCM) shown in **Figure 1*a***. In this model, orthosteric and allosteric ligands bind reversibly and saturably to their respective binding sites on the free receptor. The interaction is driven by the concentration of each ligand, their equilibrium dissociation constants (i.e., K_A, K_B) and an additional parameter, α, commonly referred to as the cooperativity factor (18). This latter parameter describes the magnitude of the allosteric change in ligand affinity that occurs between two conformationally linked sites when they are both bound and represents the ratio of the dissociation constants of the free receptor to that of the occupied receptor. Because of conformational linkage, the allosteric interaction between the two sites is reciprocal: what ligand A does to ligand B is the same as what ligand B does to ligand A. An α value >1 describes positive cooperativity (allosteric enhancement of binding), while an α value < 1 (but >0) describes negative cooperativity (allosteric inhibition of binding): an $\alpha = 1$ describes neutral cooperativity, i.e., no net effect on binding affinity at equilibrium. If it is assumed that the stimulus imparted to the cell by the ARB ternary complex is no different than the stimulus imparted by the binary AR complex, then the ATCM describes allosteric effects purely on the basis of affinity modulation.

Allosteric enhancer (or potentiator): allosteric modulators that enhance orthosteric ligand affinity and/or agonist efficacy

Allosteric antagonist (or inhibitor): allosteric modulators that reduce orthosteric ligand affinity and/or orthosteric agonist efficacy

From this model, the fractional occupancy of the receptor by orthosteric ligand A (ρ_A), in the presence of allosteric modulator, B, can be expressed as

$$\rho_A = \frac{\frac{[A]}{K_A}}{\frac{[A]}{K_A} + \frac{\left(1+{}^{[B]}/_{K_B}\right)}{\left(1+{}^{\alpha[B]}/_{K_B}\right)}}, \qquad 1.$$

where K_A and K_B denote the equilibrium dissociation constants of A and B, respectively. If α equals 0, this equation becomes one for simple competitive antagonism (19). In the absence of allosteric modulator, the occupancy of the orthosteric site is determined solely by [A] and K_A, but in the presence of modulator, the apparent equilibrium dissociation constant (K_{App}), becomes

$$K_{App} = \frac{K_A\left(1 + {}^{[B]}/_{K_B}\right)}{\left(1 + {}^{\alpha[B]}/_{K_B}\right)}. \qquad 2.$$

This relationship highlights an important feature of allosteric interactions that behave according to the ATCM, namely, that these effects are saturable, i.e., approach a limit, the magnitude of which is governed by α. Thus, as the concentration of modulator approaches infinity, the maximal allosteric effect on fractional receptor occupancy approaches the following:

$$\rho_{A[B]\to\infty} = \frac{[A]}{[A] + \frac{K_A}{\alpha}}. \qquad 3.$$

Figure 2 illustrates the effects of an allosteric enhancer ($\alpha = 10$) and an allosteric inhibitor ($\alpha = 0.1$) on the receptor occupancy of an orthosteric ligand. Compared with the theoretically infinite degree of inhibition obtained with a competitive orthosteric antagonist (**Figure 2*a***), the maximal displacement to the right of the orthosteric ligand curve by an allosteric antagonist approaches a limit (**Figure 2*b***). For the allosteric

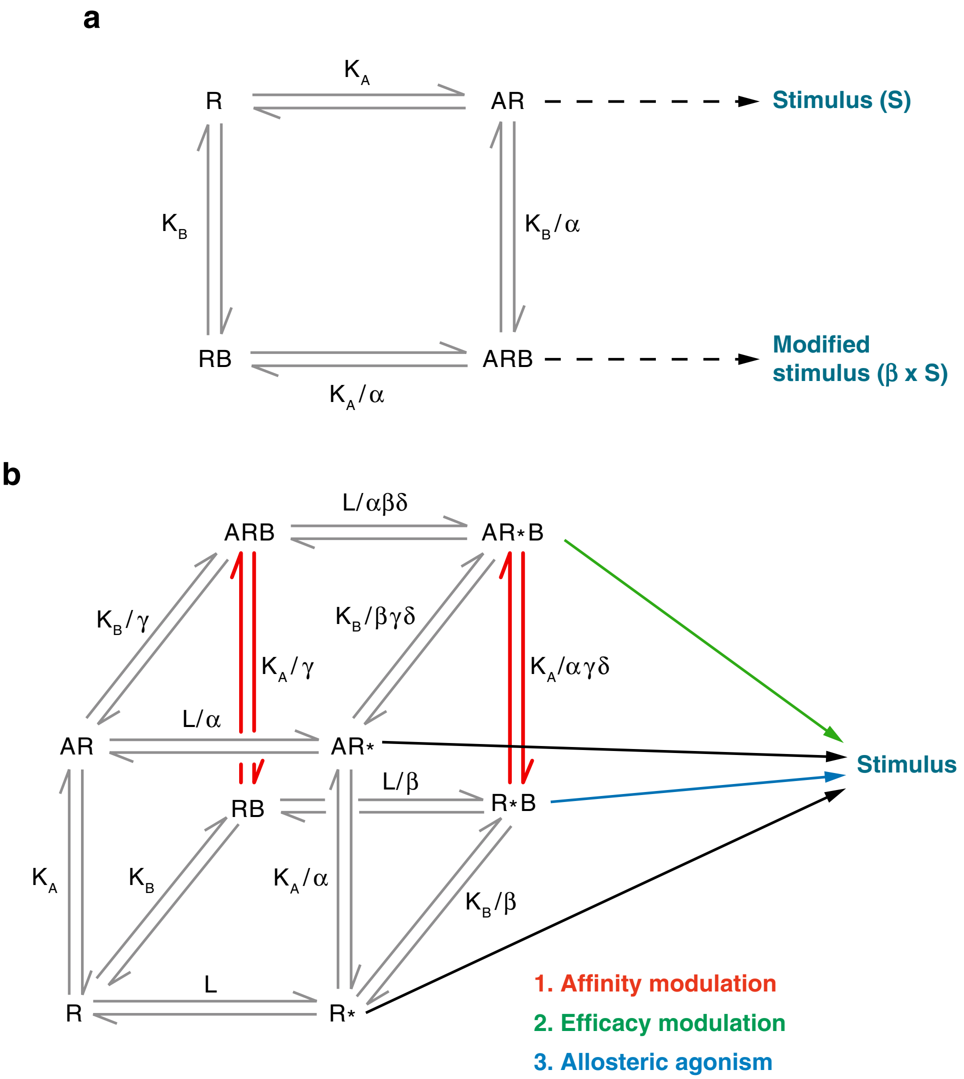
a
R
AR
K_A
Stimulus (S)
K_B
K_B/α
RB
ARB
Modified stimulus (β x S)
K_A/α
b
ARB
AR*B
$L/\alpha\beta\delta$
K_B/γ
$K_B/\beta\gamma\delta$
K_A/γ
$K_A/\alpha\gamma\delta$
AR
L/α
AR*
Stimulus
RB
L/β
R*B
K_A
K_B
K_A/α
K_B/β
L
R
R*
1. Affinity modulation
2. Efficacy modulation
3. Allosteric agonism

Figure 1

Allosteric models of drug action. (*a*) The simple allosteric ternary complex model (ATCM), which describes the interaction between an orthosteric ligand, A, and allosteric modulator, B, in terms of their respective equilibrium dissociation constants (K_A, 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 AR and ARB species, and an additional proportionality factor, β, may be added to account for modulator-induced alterations in efficacy. (*b*) The allosteric two-state model, which describes allosteric modulator effects on affinity, efficacy, and the distribution of the receptor between active (R*) and inactive (R) states, in terms of distinct conformations selected by ligands according to their cooperativity factors for the different states. Note that in this model, there are three different molecular manifestations of the allosteric effect.

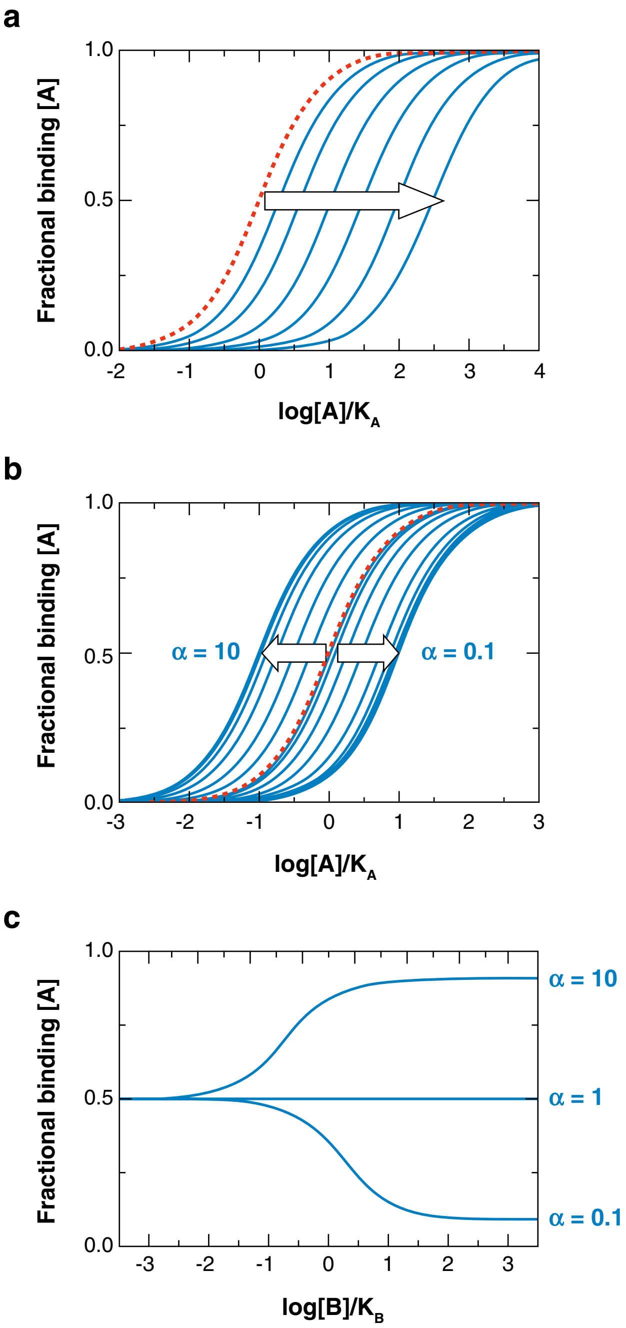

Figure 2

Behavior of the ATCM. (*a*) A competitive interaction results in a theoretically limitless rightward shift of the concentration-occupancy curve for orthosteric ligand, A. (*b*) In contrast, an allosteric enhancer ($\alpha = 10$) or allosteric inhibitor ($\alpha = 0.1$) exhibits progressive inability to maximally shift the orthosteric ligand occupancy curve at maximal modulator concentrations. Arrows indicate increasing concentrations of ligand B. (*c*) The same interactions as shown in panel *b*, but using a fixed (K_A) concentration of orthosteric ligand and increasing concentrations of either a positive, negative, or neutral allosteric modulator.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

enhancer, the orthosteric occupancy curve is shifted to the left, such that the same effect is obtained at lower concentrations of orthosteric ligand (**Figure 2*b***). However, as with the allosteric antagonist, the degree of enhancement approaches a limit defined by α. **Figure 2*c*** shows the same modulator interactions but from the point of view of a single (K_A) concentration of orthosteric ligand tested against increasing concentrations of modulator. These latter curves show the saturability of the allosteric effect at the asymptotes of the curves (determined by Equation 3).

The simulations in **Figure 2** illustrate additional aspects of the ATCM that need to be considered when applying this model to experimental data. First, as evidenced by the lack of effect on the maximal occupancy by orthosteric ligand in the presence of modulator (**Figure 2*b***), the ATCM does not predict effects on the maximal binding capacity (B_{max}) of orthosteric ligands. Second, as shown in **Figure 2*c***, the ATCM predicts monophasic, sigmoid curves with Hill coefficients of unity for both positively and negatively cooperative interactions. This contrasts with another application of the ternary complex model, namely, in describing the interaction of an orthosteric ligand, a GPCR, and a G protein (11). The main difference between the two models is that the G protein TCM can result in shallow and biphasic binding curves if the G protein amount is limiting, whereas the ATCM does not because exogenous allosteric modulator ligands are present in vast excess relative to the concentration of receptor. In addition, the ATCM does not distinguish between ligands that do or do not possess efficacy, whereas the cooperativity factor in the G protein TCM is an index of orthosteric ligand efficacy (11).

Although it is a relatively simple scheme, the ATCM highlights the fact that for drug discovery focusing on allosteric modulators of GPCRs, one needs to determine a minimum of two thermodynamic parameters, K_B and α, in contrast to orthosteric interactions, which can be characterized on the basis of K_B values alone. Moreover, K_B and α in the ATCM are independent, implying that they can be individually manipulated in structure-activity studies to attain pharmacological selectivity either on the basis of binding affinity or cooperativity with the orthosteric site.

Allosteric Effects on Efficacy

The ATCM has been successfully applied to a number of experimental systems to derive quantitative estimates of modulator affinity and binding cooperativity. Although the most direct application of this model has been to binding data, it can also be applied to functional data, but requires the additional assumption that the modulator does not perturb the signaling capacity of the receptor. Given the vast spectrum of conformational states available to GPCRs, however, it is not surprising that allosteric modulators have been discovered that affect orthosteric ligand efficacy in addition to, or independently of, effects on ligand affinity (e.g., 20–24). For example, the metabotropic glutamate mGluR1 receptor antagonist, CPCCOEt, does not affect the binding of [^{3}H]glutamate, but it concentration-dependently suppresses maximal response to glutamate in an inositol phosphate accumulation assay (**Figure 3*a***) (20); this type of allosteric antagonism is a classic example of noncompetitive antagonism. In contrast, the allosteric modulator, CGP7930, allosterically enhances both the

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

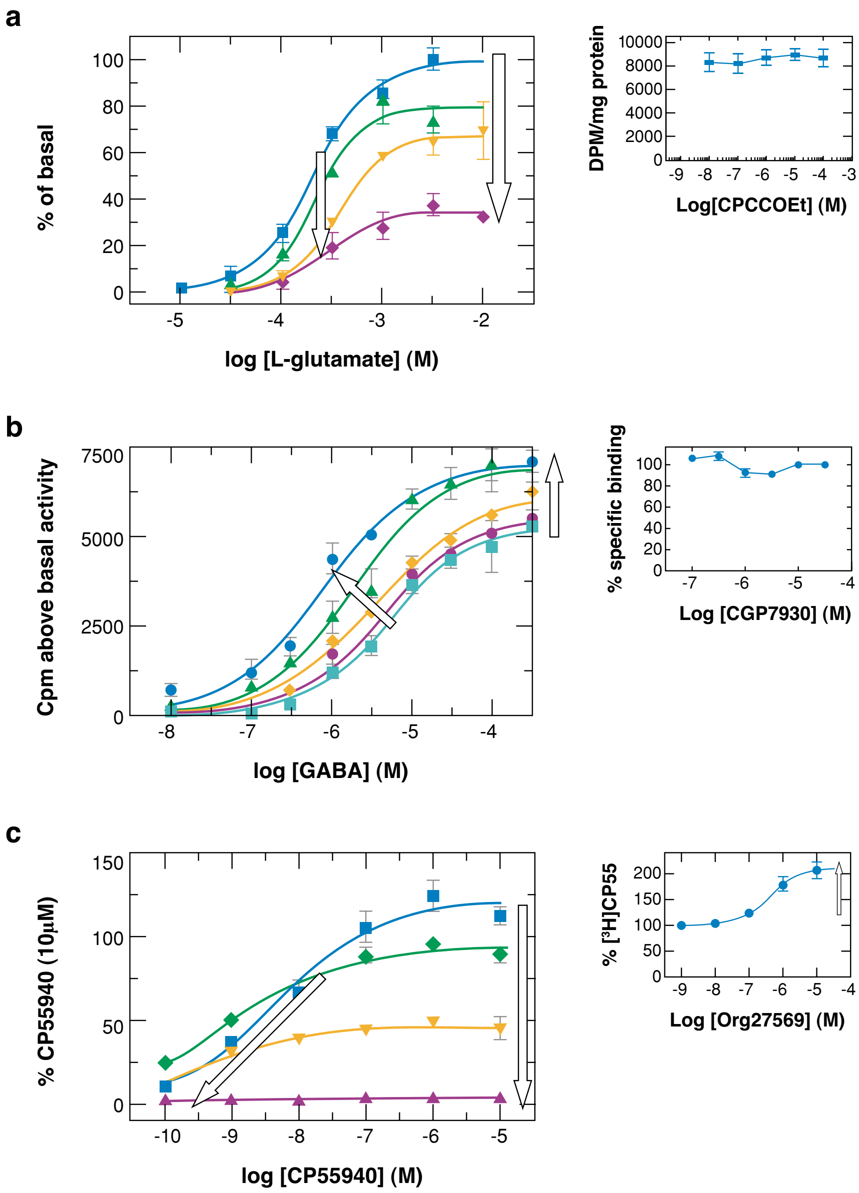

Figure 3

Allosteric effects on efficacy. (*a*) CPCCOEt reduces glutamate signaling without affecting glutamate potency or binding at mGluR1 receptors (*right column graph*); data replotted from Reference 20. (*b*) CGP7930 increases GABA potency and maximal effect but does not change the binding of the orthosteric antagonist, [^{3}H]CGP62349, at GABA$_B$ receptors (*right column graph*); data replotted from Reference 21. (*c*) The allosteric modulator, Org27569, reduces the efficacy of CP55940 at human cannabinoid CB$_1$ receptors while increasing its potency and binding affinity (*right column graph*); data replotted from Reference 24.

potency and maximal agonist effect of GABA at $\mathrm{GABA_B}$ receptors (**Figure 3*b***) (21), but has no appreciable effect on the binding of the orthosteric antagonist $[^3\mathrm{H}]$CGP62349 (**Figure 3*b*** inset). Even more strikingly, in a recent study novel allosteric modulators of cannabinoid $\mathrm{CB_1}$ receptors were shown to have a complete divergence in their effects on agonist binding versus agonist function (24). As shown in **Figure 3*c***, Org27569 is an allosteric antagonist of CP 55940 function, but allosterically enhances $[^3\mathrm{H}]$CP 55940 binding.

These types of findings are becoming increasingly reported because of the shift in emphasis from binding assays to functional assays as the high-throughput methods of choice in drug discovery (6). As a consequence, the simple ATCM becomes inadequate for describing effects of modulators that alter orthosteric ligand efficacy. To describe this in classic pharmacological terms, it is necessary to postulate an additional parameter (β in **Figure 1*a***) to account for effects of the allosteric modulator on the orthosteric agonist's intrinsic efficacy (ε). The stimulus imparted to the cell by the [AR] complex is modified by this factor when emanating from the [ARB] ternary complex (13, 24, 25). Thus, the [ARB] complex can be viewed as both a perceptive receptor species (26) because the orthosteric and allosteric ligands "perceive" each other's presence (as manifested in the parameter α), and a permissive receptor species (13), in that the stimulus imparted to the cell can be transmitted in either an unaltered or altered form (determined by both α and β).

At the molecular level, a divergence in effects of allosteric modulators on affinity relative to efficacy is consistent with the expectation that the binding of these compounds biases the conformational equilibria sampled by GPCRs to favor one set of states over another. The simplest extension of the ATCM that accommodates such properties in a conformational model is the allosteric two-state model (ATSM) of Hall (27). (**Figure 1*b***). The ATSM describes the interaction of an allosteric modulator with an orthosteric ligand on a receptor that is able to isomerize between inactive (R) and active (R^*) states. An attractive feature of this model is that it can describe both ligand efficacy and allosteric modulation in a common language, namely, cooperativity factors that modify the transition of the receptor from bound to unbound, and active to inactive, species. Thus, the cooperativity factors α and β determine the ability of the orthosteric and allosteric ligands, respectively, to promote an active receptor state; the cooperativity factor, γ, denotes the ability of each ligand to modulate the binding affinity of the other, whereas the factor, δ, denotes the ability of either ligand to modify the transition to the active state of the ternary complex. **Figure 1*b*** also highlights that the stimulus emanates from all four R^* species and that response is determined by the most abundant of these species.

From the ATSM, the fractional response of the orthosteric ligand in the presence of allosteric modulator can be expressed as

$$\frac{\mathrm{L}\left[1+\frac{\alpha[\mathrm{A}]}{\mathrm{K_A}}+\frac{\beta[\mathrm{B}]}{\mathrm{K_B}}\left(1+\frac{\alpha\gamma\delta[\mathrm{A}]}{\mathrm{K_A}}\right)\right]}{1+\mathrm{L}+\frac{[\mathrm{B}]}{\mathrm{K_B}}(1+\beta\mathrm{L})+\frac{[\mathrm{A}]}{\mathrm{K_A}}\left[1+\alpha\mathrm{L}+\frac{\gamma[\mathrm{B}]}{\mathrm{K_B}}(1+\alpha\beta\delta\mathrm{L})\right]}. \qquad 4.$$

The equation for the fractional binding of orthosteric ligand in the presence of modulator is

$$\frac{\frac{[\mathrm{A}]}{\mathrm{K_A}}}{\frac{[\mathrm{A}]}{\mathrm{K_A}} + \left[\frac{1+\mathrm{L}+\frac{[\mathrm{B}]}{\mathrm{K_B}}(1+\beta \mathrm{L})}{1+\alpha \mathrm{L}+\frac{\gamma[\mathrm{B}]}{\mathrm{K_B}}(1+\alpha\beta\delta \mathrm{L})}\right]}. \qquad 5.$$

Because Equations 4 and 5 are not identical, it follows that a functional readout can provide a different answer than a binding-based assay of receptor activity because the two formats sample different receptor species. The fact that allosteric modulators can have diverse effects on both affinity and efficacy has important implications for modulator-based drug discovery. For instance, novel ligands should ideally be characterized in as wide a spectrum of assays as possible. In addition, modulators that affect orthosteric ligand efficacy may not manifest changes in maximal agonist responsiveness if the receptor reserve of the bioassay system is high. In the presence of significant stimulus-response coupling, efficacy enhancers would not increase effects of maximal agonist concentrations above the existing ceiling, and allosteric inhibitors may not be able to reduce the maximum response over a limited range of inhibitor concentrations. Under these circumstances, the only manifestations of the allosteric effect would be an increase or decrease, respectively, in orthosteric agonist potency; such a pattern may be misinterpreted as an affinity-only allosteric effect.

Allosteric Agonism

Another important feature of the ATSM is the prediction that allosteric modulators can enrich active-state GPCR conformations in their own right, i.e., the modulators may express the property of agonism in the absence of bound orthosteric ligand. Evidence for this phenomenon was provided as far back as 1990, when Bruns & Fergus (28) demonstrated positive allosteric modulation of the adenosine A_1 receptor. In addition to enhancing the binding of the orthosteric agonist radioligand [^{3}H]cyclohexyladenosine, the allosteric modulator PD 81723 was able to inhibit forskolin-stimulated cAMP in A_1 receptor-expressing FRTL-5 cells, a classic $G_{i/o}$-mediated response characteristic of A_1 receptor activation; because this effect extends to other A_1 allosteric enhancers, the phenomenon may be a general one for this class of compounds (29). Allosteric agonists have also been reported for M_1 and M_2 mAch receptors, 5HT$_2$ receptors, chemokine CXCR4 receptors, GABA$_B$ receptors, mGluR5 receptors, mGluR7 receptors, and ghrelin receptors (6, 30–35). In addition, Zn^{2+} ions can function as natural allosteric agonists of melanocortin MC1 and MC4 receptors, as well as positive allosteric enhancers of the function of the endogenous agonist, α-MSH (36).

Intuitively, one may expect that the enrichment of an active GPCR state by an allosteric modulator would lead to both allosteric agonism and allosteric enhancement of orthosteric agonist binding/function. Similarly, the promotion of inactive receptor states could lead to allosteric inverse agonism and allosteric antagonism of orthosteric agonist binding/function. Although possible, this need not be the case. For

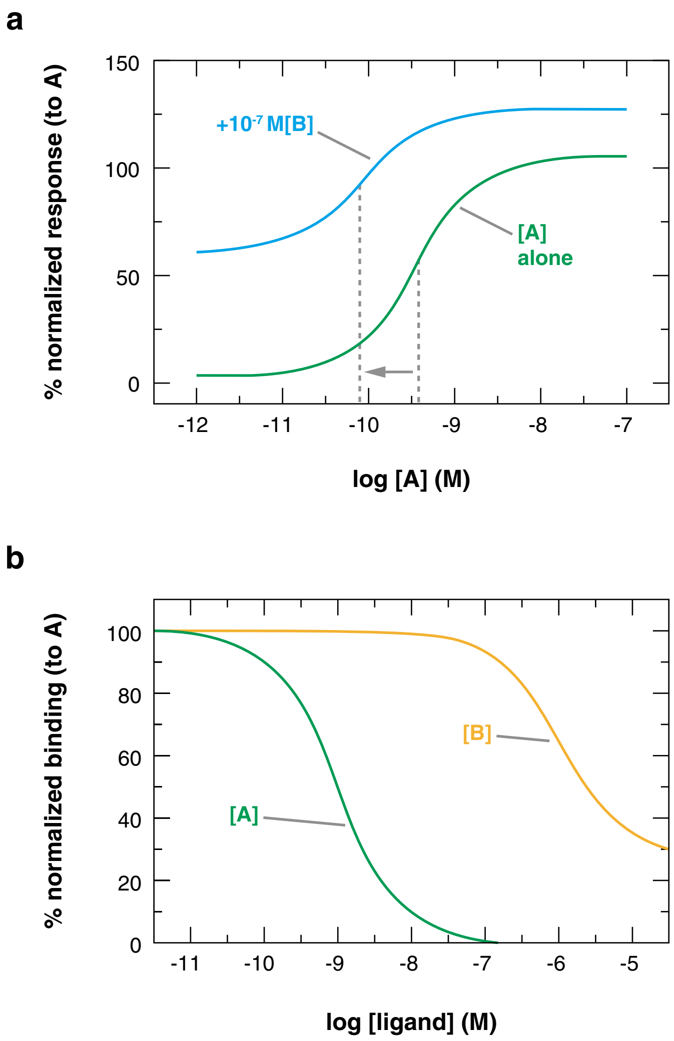

Figure 4

When binding and function do not agree. (*a*) Simulations of an allosteric agonist, B, that is able to increase both the potency and maximal agonist effect of orthosteric ligand, A, in a functional assay. Equation 4 (defined as the fractional stimulus, S) was processed through a hyperbolic stimulus-response function ($E_{max} \times S/[S + K_E]$) to account for nonlinear stimulus-response coupling; $E_{max} = 130$; $K_E = 0.0011$. (*b*) The same ligands as in panel *a*, but this time against the binding (using Equation 5) of a K_A concentration of ligand A. For all simulations, the following parameters were used: $L = 10^{-4}$, $\text{Log}K_A = -9$, $\text{Log}\ K_B = -6$, $\alpha = 30$, $\beta = 100$, $\gamma = 0.15$, $\delta = 3$.

example, although the negative allosteric modulator of mGluR5 receptors, MPEP, is an inverse agonist and an allosteric antagonist of glutamate function (37), it produces no discernible effect on the binding of glutamate. Similarly, alcuronium is an allosteric inverse agonist at M_2 mAch receptors, a weak allosteric enhancer of pilocarpine binding to the orthosteric site, but an allosteric inhibitor of pilocarpine's efficacy (22). **Figure 4** illustrates a series of simulations based on the ATSM for a modulator that exhibits negative binding cooperativity with an orthosteric agonist ($\gamma < 1$) but positive efficacy ($\beta > 1$) and positive activation cooperativity ($\delta > 1$). It can be seen that a divergence in behaviors arises depending on the measured endpoint (function versus binding): the functional assay reveals enhanced orthosteric agonist potency and maximal response whereas the binding assay reveals weak antagonism. This type of behavior has been noted for the allosteric modulator, L-692429, at ghrelin receptors (34).

Recently, Schwartz & Holst (38) have coined the term ago-allosteric modulator to differentiate allosteric ligands that display both allosteric agonism and an ability to allosterically modulate the binding and/or function of orthosteric ligands, from allosteric ligands that display only agonism or modulation. However, a consideration of the molecular mechanisms of allosterism suggest that the ago-allosteric property is theoretically present in all allosteric ligands because they all cause a redistribution of conformationally linked receptor states, which can include signaling species. The majority of allosteric agonists identified to date also can allosterically modulate (either in a positive or negative fashion) the activity of orthosteric ligands. In contrast, there are many allosteric modulators that do not appear to act as agonists; this reflects the fact that these compounds very rarely sample the conformational space that encompasses signaling receptor states, or that experiments to detect the manifestation of the agonistic property have not been performed.

More Complex Allosteric Models

In the absence of detailed structural data on the networks of amino acids that transmit allosteric effects, it is inevitable that allosteric behaviors will be observed experimentally that cannot easily be explained by relatively simple mass-action schemes such as the ATCM and ATSM. These models can be extended in efforts to accommodate such data. Some examples include a mixed mode of orthosteric and allosteric binding for the modulator (39), multiple allosteric sites (40), the addition of G protein–coupling to the ATSM (6), and the incorporation of allosteric modulation occurring across dimeric arrays (6, 41). These extensions can generally only be related to experimental data by simulation rather than fitting, but their heuristic nature can prove useful for informing the design of additional experiments.

In some cases, the apparent need to extend a model to accommodate experimental data is misguided. For example, most of the models described above do not explicitly incorporate nonlinear stimulus-response coupling, even though such coupling characterizes practically all receptor systems (19). Failure to add such a coupling step may be one reason why simpler models that relate linearly the generation of active receptor species to response apparently fail to simulate or fit experimentally observed data. Another important point is that all these models assume an equilibrium state between reactants. In reality, many biological assays, especially those used for high-throughput functional screening, are not at equilibrium over the time course of response generation and measurement. Such nonequilibrium artifacts can introduce complexity to simple orthosteric interactions (42), let alone to allosteric interactions, which by their very nature may perturb the time taken to attain equilibrium. Thus, although a mechanistic framework is absolutely indispensable for the study of allosteric mechanisms, it is necessary to ensure that the assumptions underlying the chosen model are also satisfied experimentally.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

THERAPEUTIC ADVANTAGES OF ALLOSTERIC MODULATORS

The actions of drugs that act via the orthosteric site on GPCRs rely on the affinity of the compound for the receptor of interest. To observe a therapeutic effect, an orthosteric compound must thus have a sufficiently high affinity and be maintained at a sufficiently high concentration in the receptor compartment. Under these conditions, orthosteric agonists will induce an activated state, orthosteric antagonists will induce an inactive state, and responses to both types of ligands will generally be unresponsive to fluctuations in the levels of the endogenous orthosteric ligand, even though the latter phenomenon is a normal property of physiological signaling and reflects the vital role that timing and location play in chemical transmission. As a consequence, orthosteric ligands may be associated with effects related to toxicity, desensitization, and long-term changes in receptor up/down regulation. In contrast, allosteric modulators offer the potential to overcome such responses, as well as possessing additional advantages not attainable with orthosteric ligand therapies.

The type of therapeutic advantage obtained with an allosteric modulator generally depends on the nature of the cooperative effect(s) that the modulator produces. One exception to this rule is the selectivity that can arise from binding to an allosteric site. It is possible that allosteric sites are binding sites for unidentified endogenous ligands [i.e., "orphan" allosteric sites (15)] but, by and large, this need not be the case. In contrast to orthosteric sites, therefore, allosteric sites need not have evolved to accommodate an endogenous ligand, and may simply represent domains that serve normal structural roles; only with the advent of synthetic, exogenous molecules have these domains yielded novel binding cavities for such compounds. These serendipitous allosteric sites (15) can thus show much higher sequence divergence across receptor subtypes compared to orthosteric domains owing to a lack of evolutionary pressure. This is particularly useful for GPCRs where selective orthosteric therapy has been difficult because of sequence conservation of the orthosteric site across receptor subtypes. **Figure 5*a*** shows an example of the type of selectivity that can be attained on the basis of selective binding by allosteric modulators at adenosine A_3 GPCRs, relative to A_1 and A_{2A} GPCRs (43).

Another type of pharmacological selectivity that is unique to allosteric modulators is based on cooperativity. An allosteric modulator may display neutral cooperativity ($\alpha = 1$ in the ATCM) with an orthosteric ligand at all subtypes of a given receptor except the subtype of interest, which is termed absolute subtype selectivity (44). **Figure 5*b*** demonstrates a striking example of this effect in which the allosteric modulator, thiochrome, enhances the affinity of acetylcholine at M_4 mAch receptors while having no effect at all other muscarinic (mACh) receptor subtypes. This selective effect is totally attributable to cooperativity because thiochrome binds to all five mAch receptor subtypes with almost identical affinity (44).

Given that allosteric sites can provide for selective targeting of receptors, it is also conceivable that a hybrid approach, whereby an orthosteric ligand is linked to an allosteric ligand via an appropriate spacer moiety, may provide another means of targeting the orthosteric site while using the allosteric site as a selective attachment point.

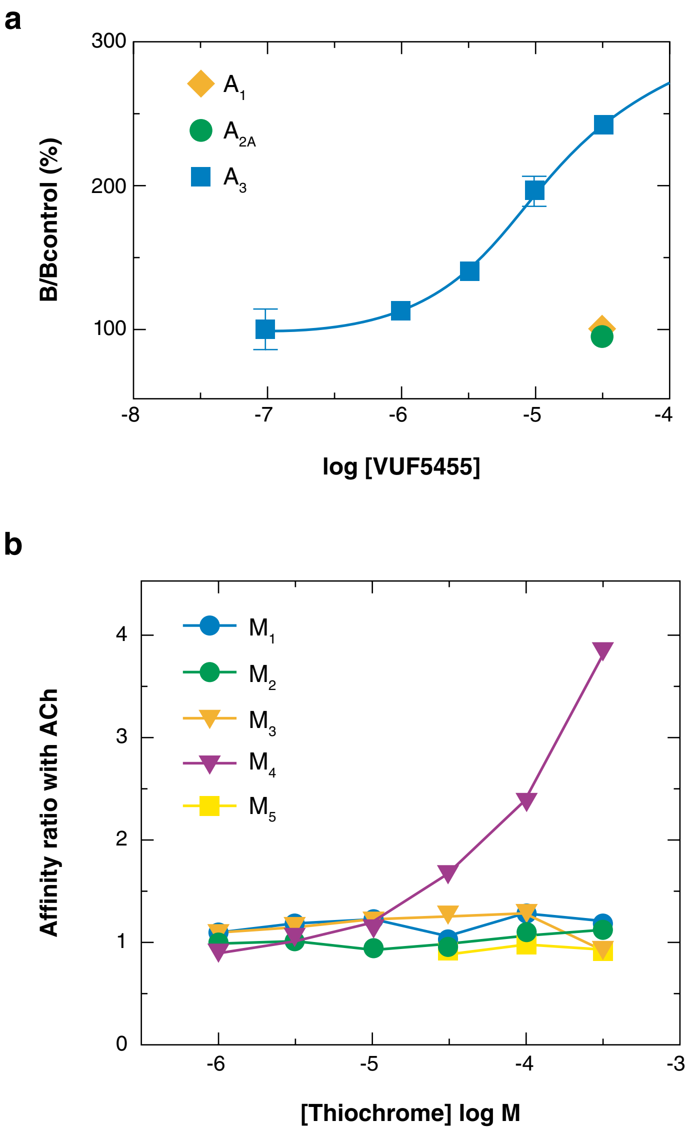

Figure 5

Modes of selectivity with allosteric modulators. (*a*) VUF5455 has selective affinity for the A_3 adenosine receptor to enhance the binding of the agonists, $[^{125}I]$-AB-MECA, compared to its lack of effect on the binding of orthosteric agonists of A_1 and A_{2A} receptors; data taken from Reference 43. (*b*) Thiochrome has similar affinity for all 5 mAch receptor subtypes, but neutral cooperativity for all subtypes except the M_4 receptor, where it selectively enhances the binding of acetylcholine (ACh) via positive cooperativity; data replotted from Reference 56.

The advantages of bivalent ligands comprising dual orthosteric pharmacophores have been known for some time (45), but very little is known about this approach with respect to orthosteric/allosteric hybrids. Recently, however, Holzgrabe and colleagues have demonstrated proof-of-concept of this method by linking the nonselective mAch orthosteric agonist oxotremorine-M to different allosteric ligands of mAch receptors (46); the results indicated that subtype-selective antagonism or even agonism could be achieved. **Figure 6*a*** illustrates schematically a mechanism for a bivalent ligand, B, that is able to occupy both orthosteric and allosteric sites on a receptor. The fractional

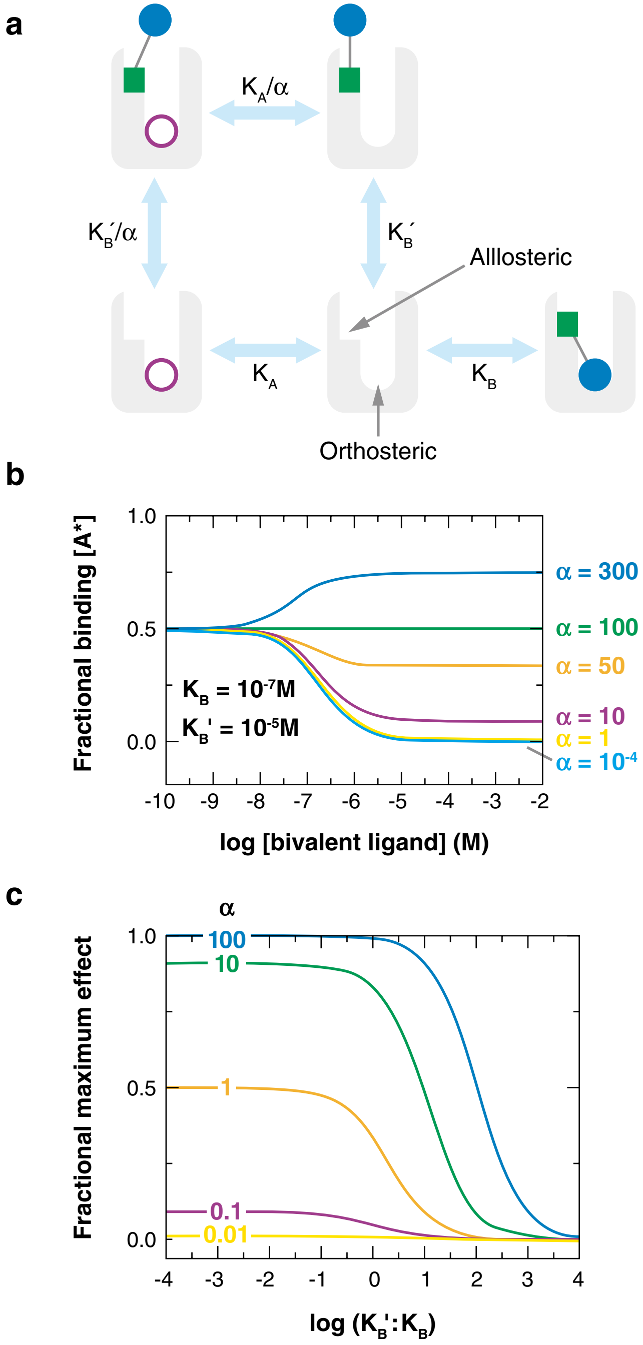

Figure 6

(*a*) A model of bivalent ligand binding to both orthosteric and allosteric sites. The orthosteric probe ligand is indicated as an open circle. (*b*) Simulations of the effects of a bivalent modulator under conditions of varying degrees of cooperativity between its allosteric moiety and an orthosteric ligand. Simulations were according to Equation 6. (*c*) Relationship between the maximum fractional effect of a bivalent modulator on the binding of an orthosteric ligand (Equation 7) and the ratio of the modulator's affinity for the allosteric site alone to its affinity for occupying both orthosteric and allosteric sites.

occupancy of orthosteric ligand, A, is given by the following equation:

$$\rho_{\mathrm{A}} = \frac{[\mathrm{A}]}{[\mathrm{A}] + \mathrm{K_A}\frac{\left(1+[\mathrm{B}]\left(\frac{1}{\mathrm{K_B}}+\frac{1}{\mathrm{K'_B}}\right)\right)}{\left(1+\frac{\alpha[\mathrm{B}]}{\mathrm{K'_B}}\right)}}, \qquad 6.$$

where $\mathrm{K_B}$ denotes the equilibrium dissociation constant of B when it occupies both orthosteric and allosteric sites, $\mathrm{K'_B}$ denotes the equilibrium dissociation constant of B when its allosteric modulator moiety occupies the allosteric site only, and α denotes the cooperativity factor for the allosteric interaction between A and B. If B does not bind to the allosteric site its affinity is governed only by $\mathrm{K_B}$, and the above equation reduces to the classic Gaddum equation for competitive antagonism. If the binding of B does not overlap with the orthosteric site, then the interaction is governed only by $\mathrm{K'_B}$ and α, and Equation 6 is equivalent to Equation 1 describing the simple ATCM. However, the overall behavior of a bivalent compound most likely represents a composite of both effects.

In general, bivalent ligands tend to show a higher affinity for the receptor than their respective component pharmacophores (45). Thus, it is reasonable to expect that $\mathrm{K_B}$ will usually be less than $\mathrm{K'_B}$ (i.e., higher affinity for dual-site occupancy versus allosteric-site occupancy alone) in the scheme shown in **Figure 6*a***, resulting in an overall inhibitory effect of the bivalent ligand on the binding of an orthosteric probe. However, this inhibition of binding may be offset by the allosteric actions of the bivalent ligand if the value of α is significantly greater than 1. **Figure 6*b*** illustrates this situation. Although the bivalent ligand has a 100-fold greater affinity when occupying both orthosteric and allosteric sites compared with its affinity for the allosteric site alone, high positive cooperativity will offset this by increasing the affinity of the bivalent ligand for the allosteric site when the orthosteric site is occupied by the probe ligand. In turn, this will result in a progressive blunting, and possibly in reversal, of the inhibitory effect of the bivalent ligand (**Figure 6*b***). This phenomenon can be explored by determining the maximal fractional effect on the binding of A, which occurs when $[\mathrm{B}] \rightarrow \infty$:

$$\rho_{\mathrm{A}_{[\mathrm{B}]\rightarrow\infty}} = \frac{[\mathrm{A}]}{[\mathrm{A}] + \frac{\mathrm{K_A}\left(1+\frac{\mathrm{K'_B}}{\mathrm{K_B}}\right)}{\alpha}}. \qquad 7.$$

When $\mathrm{K_B} \ll \mathrm{K'_B}$, the maximum fractional effect approaches 0, as expected for competitive interactions. In contrast, when $\mathrm{K'_B} \ll \mathrm{K_B}$, the maximum fractional effect on binding is determined by α, as shown in Equation 3. In between these two extremes, different effects can be observed. **Figure 6*c*** illustrates the relationship between maximal fractional effect and the ratio of $\mathrm{K'_B}$ to $\mathrm{K_B}$ for different values of α. The concentration of radioligand for the simulations was equal to its $\mathrm{K_A}$, so the initial fractional level of binding is 0.5. If the affinity of the bivalent ligand for occupying both sites is substantially increased relative to the affinity for the allosteric site alone (i.e., $\mathrm{K_B} < \mathrm{K'_B}$), then the maximal effect would be inhibitory unless the allosteric component of the interaction is characterized by very high positive cooperativity. This is consistent with the simulations shown in **Figure 6*b***, where the effect switched from inhibitory to potentiating at very high values of α. Thus, if the main purpose in the design of a bivalent compound is to target the orthosteric site (either as an agonist or antagonist) while gaining selectivity from the allosteric site, then the allosteric component of the molecule should possess negative, neutral, or only weak positive cooperativity.

In addition to novel modes of selectivity, the saturability of allosteric interactions also represents a therapeutic advantage, because it leads to a ceiling over and above which further increments in modulator dose will not produce target-based effects. This phenomenon is governed by the cooperativity factor of the interaction and thus relies on the modulator possessing limited degrees of cooperativity. By and large this applies to all allosteric enhancers, but many allosteric antagonists exhibit very high degrees of negative cooperativity with the endogenous agonist such that their behavior is indistinguishable from that of a competitive antagonist. Where applicable, however, saturability in the allosteric effect has distinct advantages. Specifically, the modulator may be administered in a larger dose than would be applied to an orthosteric ligand of similar affinity, thus maintaining an adequate depot in the receptor "compartment" without the dangers normally associated with high doses of orthosteric ligands. Therefore, allosteric modulators can be more forgiving with respect to affinity requirements than orthosteric ligands for at least two reasons: 1) low-affinity modulators can be given in larger doses while maintaining a favorable (mechanism-based) safety profile, and 2) the cooperativity factor may compensate for the low affinity and be the primary determinant of the selectivity, as well as the magnitude, of the effect.

If an allosteric modulator does not possess appreciable efficacy, it can provide another powerful therapeutic advantage over orthosteric ligands, namely, the ability to selectively tune up or down tissue responses only when the endogenous agonist is present. This provides a spatial and temporal character to the actions of such modulators that is unattainable with orthosteric ligands. There are a number of circumstances where this property can prove particularly useful. For example, neurodegenerative disorders such as Alzheimer's disease are characterized by suboptimal therapies with respect to orthosteric ligands (47). Allosteric enhancers may provide superior alternatives, because they will boost the actions of the endogenous neurotransmitter and thus maintain the spatial and temporal profile associated with signaling via that transmitter.

The preceding examples mostly relate to allosteric modulators that do not possess efficacy in their own right. Allosteric agonists represent a different situation because the theoretical advantage of modulating endogenous activity only when the orthosteric site is occupied is not applicable to such agents. It is also currently unknown whether allosteric agonists will engender similar degrees of receptor desensitization, phosphorylation, internalization, etc., as observed with orthosteric ligands; Klaasse et al. (48) have recently shown that the allosteric enhancer, PD 81723, can enhance agonist-mediated internalization of the adenosine A_1 receptor while having no acute effect on its own, but May et al. (49) have shown that prolonged exposure to M_2 mAch receptor modulators can lead to an alteration of cell surface receptor expression.

There are three potential advantages to allosteric agonists. The first is that allosteric agonism may be the best way to obtain selective agonists for certain receptors, as has been seen with the M_1 mAch agonist, AC-42 (50) and the mGluR7 agonist, AMN082 (33). This can be achieved on the basis of selective affinity for a nonconserved binding site or by selective signaling; allosteric agonists, by promoting unique conformational states, may activate one type of cellular pathway (e.g., effector coupling) but not another (e.g., desensitization). The second advantage of allosteric agonists is that they may find clinical utility in combination therapies for certain disorders. For example, blockade of the orthosteric site on the chemokine CXCR4 receptor has been identified as a potential means for combating HIV, but it can lead to severe side effects. One way around this is to use allosteric agonists that are neutrally cooperative with coadministered orthosteric antagonists; proof of concept for this approach has recently been provided (30). Finally, allosteric agonists that act as enhancers of the endogenous agonist may prove clinically more efficacious compared with either agonists or allosteric enhancers alone (38).

DETECTING, VALIDATING, AND QUANTIFYING ALLOSTERIC EFFECTS

GPCR-based screening assays require a "probe" that provides a readout of receptor activity. One such physiological probe is the G protein itself; coupling of constitutively active receptors to G proteins, for instance, can provide a signal susceptible to perturbation by test ligands. Usually, however, the assay is supplemented with a chemical probe, ideally the endogenous agonist for that receptor or a surrogate agonist, against which the effects of test compounds are compared. Similarly, binding assays use labeled agonist or antagonist probes. Because allosteric interactions are characterized by cooperativity between topographically distinct sites, the nature of the probe can make a huge difference in one's ability to detect an allosteric effect; each combination of orthosteric probe and allosteric ligand will be characterized by unique cooperativity factors. Thus, a characteristic feature of allosteric interactions is that they are probe-dependent. A second characteristic, as discussed above, is that allosteric effects can be saturable, such that further increments in modulator concentration do not produce further effect. These two characteristics of allosteric interactions are the main signposts that should guide discovery efforts toward the detection, validation, and quantification of an allosteric effect. Ideally, such studies should be supplemented, where possible, with more direct evidence of the ability of a GPCR to bind more than one ligand (see below).

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

Radioligand Binding Assays

Although assays of GPCR function have surpassed binding assays as the high-throughput screening methods of choice, binding assays still retain importance in studies of GPCR allosterism because they can, in many cases, directly validate an allosteric mode of action. The need to differentiate allosteric from orthosteric ligands often requires that ligands be characterized on the basis of where they bind, in addition to what they do. Depending on the cooperativity factor, α, allosteric antagonists can be revealed by their inability to fully inhibit the specific binding of a radiolabeled orthosteric probe (**Figure 2*c***). However, this may not always be readily apparent. The detection of allosteric effects requires careful consideration of the signal-to-noise window, which depends primarily on two factors: the concentration of probe used in the assay and the cooperativity between the probe and the allosteric modulator. As shown in **Figure 7**, changing the concentration of orthosteric probe will produce characteristic effects on the maximal asymptotic level of binding (governed by the relationship shown in Equation 3) and the midpoint location parameter ($[B]_{50}$) of the enhancement (**Figure 7*a***) or inhibition (**Figure 7*b***) curve. Under these conditions $[B]_{50}$ is defined by the following relationship (6):

$$[B]_{50} = K_B \left(\frac{[A] + K_A}{\alpha[A] + K_A} \right). \qquad 8.$$

Equations 3 and 8 have important implications for the detection of allosteric modulators. First, the allosteric interaction may not be readily discernible if the concentration of orthosteric probe is too high. Second, at low concentrations of orthosteric probe, allosteric antagonists may appear indistinguishable from orthosteric antagonists, especially if the negative cooperativity is high. Third, a determination of $[B]_{50}$ as a function of radioligand concentration can be a useful measure for differentiating competitive antagonism from allosteric inhibition. Whereas a simple competitive interaction is characterized by a linear relationship between occupancy and shift (the latter defined by the midpoint location parameter, IC_{50}), allosteric antagonism yields a curvilinear relationship, which becomes more pronounced as α approaches 1 (**Figure 7*c***). Directly labeling a high-affinity allosteric modulator and monitoring its interaction in competition with test ligands can obviate many of these issues. However, examples of labeled modulators are currently limited, and even when they are available, they still require studies that determine the type of cooperativity that occurs with orthosteric ligands.

Another useful application of binding assays to detect and quantify allosteric interactions involves the study of allosteric effects on the rates of association or dissociation of orthosteric ligands with the GPCR. As noted above, affinity modulators must invoke a conformational change that alters either one or both kinetic rate constants that govern the binding of the orthosteric ligand. The most common investigational method is to assay dissociation kinetics because the only way dissociation of a prebound GPCR-orthosteric ligand complex can be modified is by the concomitant binding of a modulator to a topographically distinct and vacant site. Although association kinetic assays can be, and have been, used to detect allosteric effects, a second orthosteric ligand can alter the apparent association rate of an orthosteric ligand owing to competition between the two ligands that affects the time required to achieve a new equilibrium (51).

Effects of allosteric modulators on orthosteric ligand dissociation have been detected for all three major subclasses of mammalian nonsensory GPCRs. In some cases, the effect is an acceleration of dissociation, whereas in others it is a slowing. Analytical methods have also been developed to quantify these effects according to the ATCM (52). These methods are very useful in providing an internal check for the ATCM as an appropriate model for a given allosteric interaction because the

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

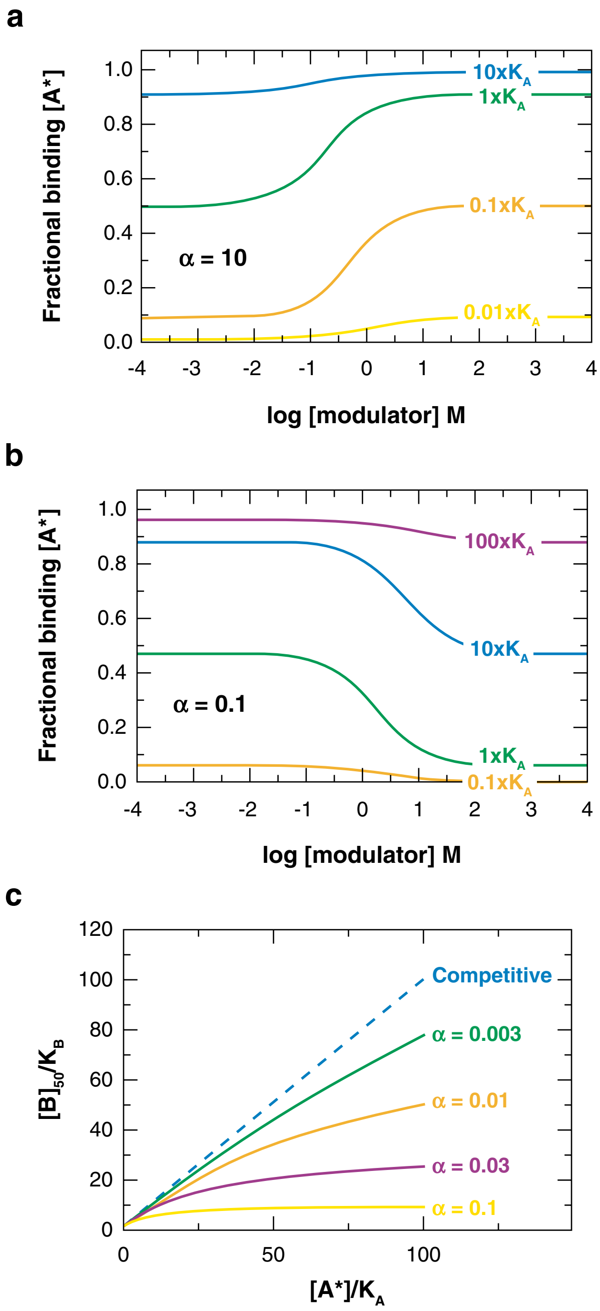

Figure 7

Detecting allosteric effects in equilibrium binding assays. Simulations were performed according to Equation 1 for an allosteric enhancer (*a*) or inhibitor (*b*); note the effects of increasing radioligand concentration. (*c*) The increase in the midpoint location parameter for an antagonist ($[B]_{50}$) as a function of the amount of radioligand in the assay (Equation 8); data replotted from Reference 6.

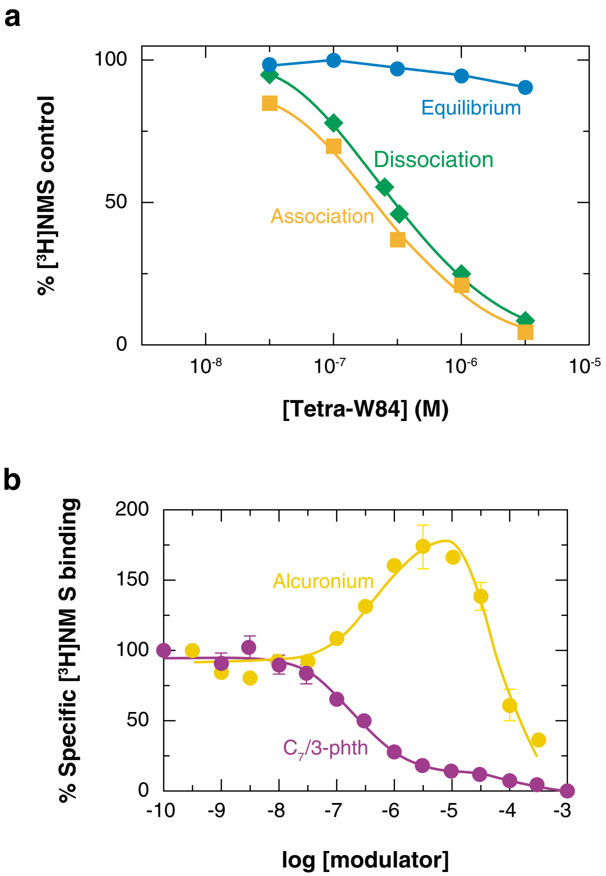

Figure 8

Kinetic effects can be used to unmask allosterism, but can also lead to complex behaviors. (*a*) The allosteric modulator, tetra-W84, displays neutral equilibrium binding cooperativity with [^{3}H]N-methylscopolamine at M_2 mAch receptors. However, its allosteric properties are revealed when its effects on association or dissociation kinetics of [^{3}H]NMS are monitored; data replotted from Reference 53. (*b*) Effects of the allosteric enhancer, alcuronium, or the allosteric inhibitor, heptane-1,7-bis-(dimethyl 3′-phthalimidopropyl)-ammonium bromide (C_7/3-phth), on the binding of [^{3}H]NMS at recombinant human M_2 mAch receptors after 90 min (alcuronium) or 60 min (C_7/3-phth) incubation. Complex binding curves are observed because the highest concentrations of each modulator retard the dissociation of the radioligand to such an extent that reaction equilibrium is not achieved within the incubation period. Data replotted from Reference 54.

results obtained from equilibrium assays should be consistent with those obtained from the kinetic assays (6, 52, 53). Another advantage of the kinetic binding assay is that it can detect allosteric modulators that may be missed in equilibrium binding studies. As shown in **Figure 8*a***, the allosteric modulator, tetra-W84, interacts with neutral cooperativity ($\alpha = 1$) against the orthosteric mAch receptor probe,

[^{3}H]N-methylscopolamine ([^{3}H]NMS). However, its allosteric nature is unmasked when its effects on [^{3}H]NMS association and dissociation kinetics are monitored (53); because the equilibrium binding affinity is defined as the ratio of ligand association to dissociation rates, and because tetra-W84 affects these to the same extent over the same concentration ranges, neutral cooperativity with [^{3}H]NMS results at equilibrium.

The ability of allosteric modulators to exert profound effects on the binding kinetics of orthosteric ligands can also lead to complex binding curves in assays that are ostensibly conducted under equilibrium conditions. A contributing factor is that the approach of a system toward an equilibrium state is limited by the dissociation rate of the slowest-dissociating component (51). If this component is the orthosteric probe, then, at high modulator concentrations, one may observe marked deviation from binding equilibrium such that it is not attained during the time course of the assay. **Figure 8*b*** shows the outcome of this effect for the binding of an allosteric enhancer, alcuronium, and inhibitor, C_7/3-phth, which both slow the dissociation of [^{3}H]NMS from M_2 mAch receptors (54). The complex curves were fitted to a kinetic version of the ATCM (52, 54) to derive appropriate estimates of K_B and α, but it is obvious that a lack of appreciation of the kinetic effects of modulators can erroneously lead one to seek alternate and more complex models to accommodate the data.

There are examples of allosteric ligands that appear to exert profound effects on the B_{max} of orthosteric ligands, with or without effects on orthosteric ligand affinity (e.g., 23, 55, 56). Although there are a number of potential mechanisms underlying this phenomenon, most remain poorly explored. One possibility is that the allosteric modulator has such a slow rate of dissociation from its binding site as to be essentially irreversible. If the interaction is characterized by negative cooperativity, then the consequence of irreversible or pseudoirreversible modulator binding would be a permanent occlusion of the orthosteric ligand binding pocket. This finding is consistent with the effects of allosteric antagonists of the chemokine CCR5 receptor, such as 873140 (23), which bind in an essentially irreversible fashion. Because allosteric interactions are reciprocal, dissociation kinetic assays would not be able to detect such modulators. The readout from such assays is the radioactivity of the orthosteric ligand, which is preequilibrated with the receptor and its dissociation monitored over time; as long as the orthosteric probe is on the receptor, however, the negative cooperativity would result in occlusion of the allosteric pocket, thus preventing the modulator from ever binding and changing the dissociation of the orthosteric ligand. Under such conditions, alternative approaches are required to validate an allosteric mode of action. One possibility is to perform interaction experiments with different modulators, preferably of different chemical structures, which would be expected to blunt one another's effects if they compete for a common allosteric site (23). Moreover, these types of experiments can also be utilized to detect multiple allosteric sites that interact both with one another and with the orthosteric site with different degrees of cooperativity (57). In situations where modulator binding is readily reversible, interaction experiments can prove even more useful, as they can be applied to both kinetic and equilibrium binding assays (57, 58).

Functional Assays

Functional assays represent the primary platform for GPCR-based drug discovery. This is logical, given that the ultimate goal of pharmacotherapy is to perturb physiological function for therapeutic benefit. Advantages of functional versus radioligand binding assays include the ability to detect a wider spectrum of behaviors, with less bias placed on modulators that predominantly affect orthosteric ligand binding affinity. Functional assays can readily detect allosteric ligands that mediate their effects on orthosteric ligand efficacy as well as affinity. It is not surprising, therefore, that the advent of high-throughput functional screening has dramatically increased the number of hits, but this has also led to some difficulties in assay and lead optimization for compounds that mediate their effects through allosteric mechanisms owing to issues related to validation and potential divergence of findings when different assay formats are used (47, 59).

The simplest approach for detecting and quantifying allosteric effects in a functional assay is to investigate the effects of increasing concentrations of putative modulator ligands on the location (i.e., EC_{50}) of the concentration-response curve for an orthosteric agonist, in a manner analogous to classic Schild analysis (6). Because the most relevant interactions *in vivo* are those with the endogenous agonist, the latter should always be used in functional studies wherever possible. For some systems, e.g., orphan receptors, this may not be possible but it should be noted that the probe-dependence of the allosteric effect can lead to different results with surrogate probes relative to endogenous agonists. For allosteric antagonists that do not affect the maximum or basal responsiveness, the concentration ratio (CR) is determined by dividing the EC_{50} in the presence of antagonist to that in its absence. This ratio is related to the ATCM as follows (18):

$$\mathrm{CR} - 1 = \frac{[\mathrm{B}](1-\alpha)}{\alpha[\mathrm{B}] + \mathrm{K_B}}. \qquad 9.$$

In a similar manner, CRs can also be determined for allosteric enhancers; in this case the control agonist EC_{50} should be divided by that obtained in the presence of enhancer. This CR can be related to the ATCM as shown below (18):

$$\mathrm{CR} = \frac{\alpha[\mathrm{B}] + \mathrm{K_B}}{[\mathrm{B}] + \mathrm{K_B}}. \qquad 10.$$

Figure 9 shows the application of these equations to the analysis of the allosteric antagonist, gallamine at M_2 mAch receptors (**Figure 9*a***), and the allosteric enhancer, PD117975 at adenosine A_1 receptors (**Figure 9*b***). The saturability of the allosteric effect is manifested as a curvilinear regression of the logarithm of the modulator concentration on the logarithm of CR−1 (inhibitors) or CR (enhancers). As with the binding assay, this functional approach has certain implications for the study of antagonists. If the negative cooperativity is very high, then the regression will be difficult to distinguish from a linear Schild regression that is obtained with competitive antagonists (6). It is therefore necessary to study the actions of antagonists over as wide a concentration range as possible. The probe dependence of allosterism also predicts that application of the Schild method to allosteric antagonists can result in

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

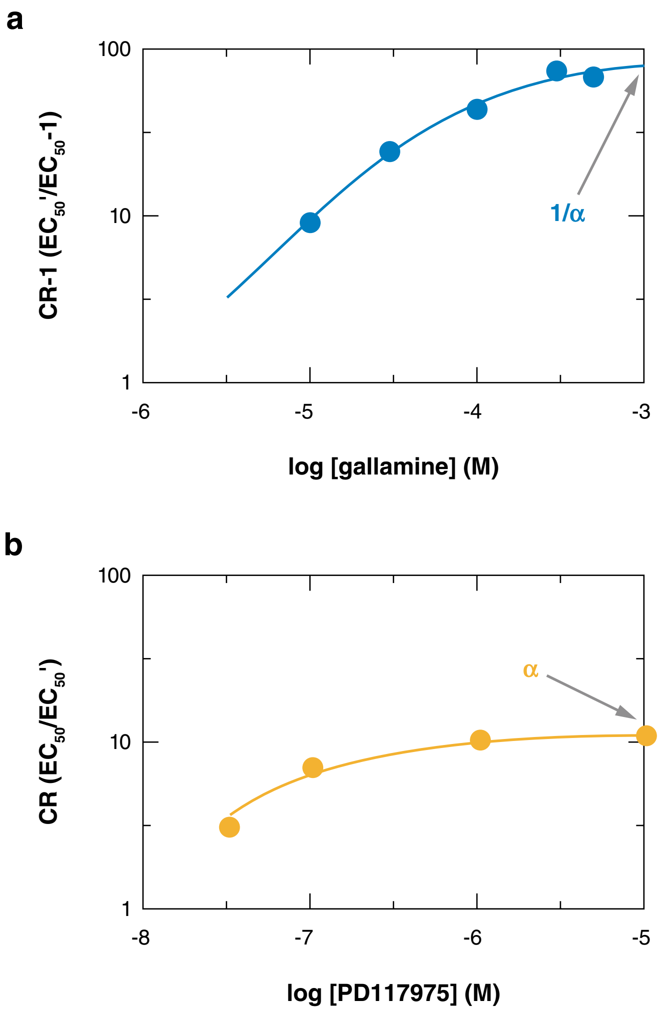

Figure 9

Schild analysis of allosteric modulator effects. (*a*) Regression (Equation 9) of the effects of increasing concentrations of gallamine as an antagonist of acetylcholine-mediated negative ionotropic effects on the electrically driven guinea pig atrium; data replotted from Reference 42. (*b*) Regression (Equation 10) of the effects of the enhancer, PD117975, on R-PIA-mediated stimulation of ERK1/2 phosphorylation at human adenosine A_1 receptors, expressed in a CHO cell line. Note the differences in the calculation of the concentration ratio (CR) for the inhibitor relative to the enhancer.

different Schild slopes and pA_2 estimates (attributable to different α values), i.e., the antagonism will be agonist-dependent, in contrast to classic orthosteric antagonism, which is agonist-independent if the same receptor mediates both the agonist and antagonist effects (13).

As with the radioligand binding assay, functional assays are commonly optimized for high-throughput screening by monitoring the effects of increasing concentrations of a test compound against a fixed concentration of orthosteric ligand. When screening for an allosteric modulator, it is important to determine the optimal concentration of agonist so as to increase the likelihood of detecting an allosteric effect while maintaining a reasonable signal-to-noise ratio. This is particularly important for modulators with limited degrees of cooperativity because the detection window will be small. One can determine the maximal window for detecting allosteric effects in functional assays by comparing the effects of different degrees of cooperativity on the concentration-response curve of an agonist at maximal occupancy of the allosteric site. The response to an agonist, A, in the presence of allosteric modulator, B, the latter of which affects only the affinity of the agonist according to the ATCM, can be modeled as a general logistic equation of the following form:

$$\mathrm{E} = \frac{\mathrm{E}_{\max} \cdot [\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{A}]^{\mathrm{n_H}} + [\mathrm{EC}_{50}]^{\mathrm{n_H}} \left(\frac{1+\frac{[\mathrm{B}]}{\mathrm{K_B}}}{1+\frac{\alpha[\mathrm{B}]}{\mathrm{K_B}}} \right)}, \tag{11.}$$

where E represents effect and $\mathrm{n_H}$ denotes the logistic slope factor for the curve. In the absence of modulator, this equation reduces to that of the simple logistic function:

$$\mathrm{E}_{[\mathrm{B}]=0} = \frac{\mathrm{E}_{\max} \cdot [\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{A}]^{\mathrm{n_H}} + [\mathrm{EC}_{50}]^{\mathrm{n_H}}}. \tag{12.}$$

At maximal occupancy of the allosteric site (i.e., $[\mathrm{B}] \rightarrow \infty$), this equation becomes

$$\mathrm{E}_{[\mathrm{B}]\rightarrow\infty} = \frac{\mathrm{E}_{\max} \cdot [\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{A}]^{\mathrm{n_H}} + \frac{[\mathrm{EC}_{50}]^{\mathrm{n_H}}}{\alpha}}. \tag{13.}$$

To determine the maximal window of agonist response for different degrees of cooperativity at each concentration of agonist, therefore, one must subtract Equation 12 from Equation 13.

$$\mathrm{E}_{[\mathrm{B}]=0} - \mathrm{E}_{[\mathrm{B}]\rightarrow\infty} = \frac{\mathrm{E}_{\max} \cdot [\mathrm{A}]^{\mathrm{n_H}} \cdot [\mathrm{EC}_{50}]^{\mathrm{n_H}}(\alpha - 1)}{([\mathrm{A}]^{\mathrm{n_H}} + [\mathrm{EC}_{50}]^{\mathrm{n_H}}) + (\alpha \cdot [\mathrm{A}]^{\mathrm{n_H}} + [\mathrm{EC}_{50}]^{\mathrm{n_H}})}. \tag{14.}$$

This can be rearranged in terms of fractional response and to normalize agonist concentrations to the EC_{50}:

$$\frac{\mathrm{E}_{[\mathrm{B}]=0} - \mathrm{E}_{[\mathrm{B}]\rightarrow\infty}}{\mathrm{E}_{\max}} = \frac{\frac{[\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{EC}_{50}]^{\mathrm{n_H}}} \cdot (\alpha - 1)}{\left(\frac{[\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{EC}_{50}]^{\mathrm{n_H}}} + 1\right) + \left(\alpha \cdot \frac{[\mathrm{A}]^{\mathrm{n_H}}}{[\mathrm{EC}_{50}]^{\mathrm{n_H}}} + 1\right)}. \tag{15.}$$

Figure 10 shows the detection windows for different degrees of cooperativity versus different concentrations of an orthosteric agonist, normalized to its EC_{50}. These simulations highlight two important points: (*a*) different fixed concentrations of orthosteric agonist provide the optimal likelihood of detecting an allosteric effect, depending on whether the screen is for enhancers or inhibitors; and (*b*) this process depends on the slope of the agonist concentration-response curve. Functional assays characterized by very steep agonist concentration-response relationships (e.g., most calcium-based assays) have stricter requirements than assays where the agonist concentration-response curve is characterized by slopes < 1.

The principles underlying concentration ratio analysis have been modified and extended over the years to accommodate a variety of approaches for studying allosterism at GPCRs. These include direct fitting of the ATCM to functional data using nonlinear regression (52), additivity of concentration ratio analyses (60), and pharmacological resultant analysis (61). However, for all these situations it is assumed that the allosteric modulator does not affect efficacy, only affinity, of the orthosteric ligand. If this is not the case, then the application of the ATCM is inappropriate

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

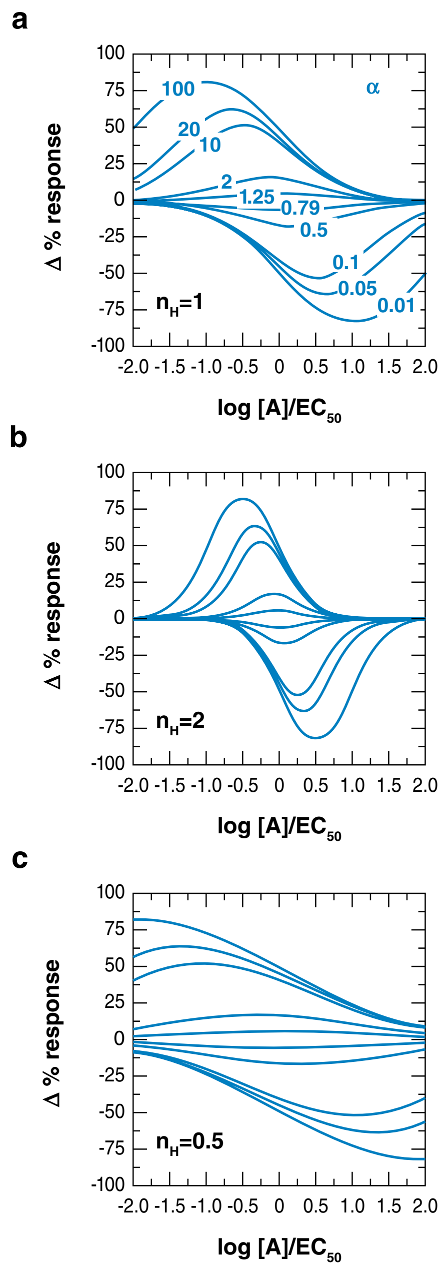

Figure 10

Detection windows for allosteric effects in functional assays. Equation 15 was used to simulate the maximal attainable allosteric effect for varying degrees of cooperativity when tested against different concentrations of an agonist (as a multiple of its EC_{50}) whose concentration-response curve is characterized by a normal (i.e., unity) (*a*), steep (*b*), or shallow (*c*) Hill coefficient (n_H).

and the method will yield erroneous estimates of the cooperativity factor, α (18). A method has recently been presented that follows these issues in greater detail (25).

The methods described above can also be applied to the detection and quantification of allosteric agonists. Thus, the agonist should be tested against as wide a range as possible of concentrations of orthosteric antagonist. If the Schild regression deviates from linearity, this may indicate negative cooperativity. This method was recently used to demonstrate that the agonist, AC-42, was interacting allosterically with the orthosteric antagonist, atropine, at mAch M_1 receptors (35). Furthermore, the probe-dependence of the effect was further validated when different degrees of antagonism were observed with another orthosteric antagonist, pirenzepine (35). Unfortunately, this approach will only work if there is appreciable cooperativity between the allosteric agonist and orthosteric antagonist. The probe-dependence of allosterism means that certain allosteric agonists may interact with essentially neutral cooperativity with the chosen orthosteric antagonist, thus manifesting an apparent resistance to antagonism (30, 33).

Given the widespread use of functional screening assays, allosteric modulators are now being identified that affect orthosteric ligand efficacy (see above). The quantification of such effects requires extension of the ATCM, which only deals with effects on affinity. Although a molecular model such as the ATSM (**Figure 1*b***; Equations 4 and 5) describes allosteric effects on efficacy, it contains many parameters that cannot readily be fitted to concentration-response data unless certain assumptions regarding the degree of receptor reserve and constitutive receptor activity of the system are met. An alternative approach is to derive an operational model of allosterism that contains as few parameters as possible while allowing for experimental quantification of allosteric effects on affinity and efficacy. One such model that has been derived (13, 24, 25), utilizes a simple extension of the ATCM and includes an additional empirical proportionality constant, β (**Figure 1*a***), which accounts for allosteric effects of the modulator on orthosteric ligand intrinsic efficacy. The derivation of the model is described elsewhere (24) but one form applicable to experimental concentration-response data is:

$$E = \frac{E_m \tau^n [A]^n \left(1 + \frac{\alpha\beta[B]}{K_B}\right)^n}{\left[[A]\left(1 + \frac{\alpha[B]}{K_B}\right) + K_A\left(1 + \frac{[B]}{K_B}\right)\right]^n + \tau^n [A]^n \left(1 + \frac{\alpha\beta[B]}{K_B}\right)^n}, \qquad 16.$$

where E denotes the effect, E_m denotes the maximum possible effect, τ is an operational measure of orthosteric ligand efficacy equal to the product of the intrinsic efficacy and maximum number of receptors, and n is a logistic slope factor that governs the shape of the stimulus-response function. **Figure 11** shows the application of this model to the interaction between the allosteric antagonist, Org27569, and the agonist WIN55212-2 at CB_1 receptors in a mouse vas deferens preparation (24), and to the allosteric enhancer, GS39783, against GABA at recombinant $GABA_B$ receptors (62). In the case of Org27569, the model estimated an α value greater than 1, indicating positive binding cooperativity (confirmed in separate binding assays), but a β value less than 1, accounting for the diminution in efficacy. For the

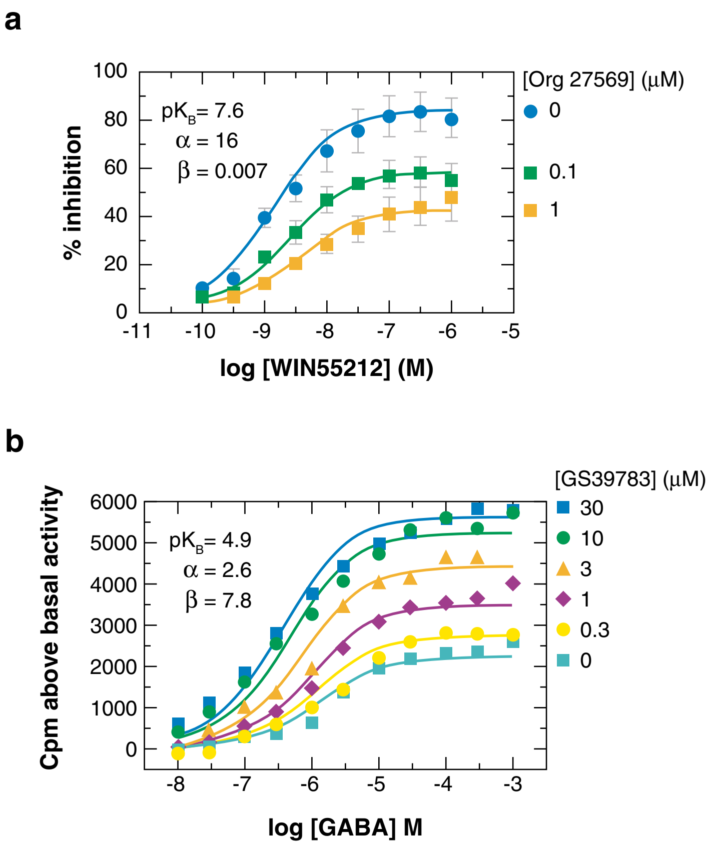

Figure 11

An operational model of allosterism. (*a*) The CB$_1$ modulator Org27569 is an allosteric antagonist of WIN55212-mediated effects on the electrically evoked contractions of the mouse vas deferens; data replotted from Reference 24. (*b*) GS39783 is an allosteric enhancer of the effects of GABA at GABA$_B$ receptors in a cell-based assay of inositol phosphate accumulation; data replotted from Reference 62. In both panels, the datasets were fit to Equation 16 to derive estimates of the negative logarithm of the allosteric modulator dissociation constant (pK$_B$), the effect on orthosteric ligand binding (α), and the effect on orthosteric ligand efficacy (β).

GABA$_B$ enhancer, the model reveals modest positive cooperativity on the binding of GABA, and a more pronounced effect on the signaling of the orthosteric ligand. Thus, this operational model of allosterism represents a minimalist model that can be fitted to experimental data to help inform the design of additional structure-function studies.

Other Methods

In studies of enzymes, allosteric modulators have routinely been detected by high-throughput screening using enzyme kinetic assays and in a number of cases followed by X-ray crystallography to confirm the site of action of a modulator as a pocket distinct from the enzyme's active site. Specific examples of this approach include the discovery of inhibitors for HIV-1 reverse transcriptase, p38 MAP kinase, and protein tyrosine phosphatase 1B, and of allosteric activators for the enzyme glucokinase (15). Unfortunately, thus far, there is only one published crystal structure of a GPCR, e.g., bovine rhodopsin in its inactive state (63), and structure-based in silico screening approaches will require further refinement before proving robust enough to supplant

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

functional screening for modulators as the method of choice. However, a number of computational advances in recent years may provide new methods for identifying allosteric pockets on proteins. For example, Abagyan and colleagues have developed a novel algorithm, internal coordinate mechanics, which has successfully identified novel binding pockets in a diverse range of proteins (including GPCRs), the so-called pocketome (64, 65).

Additional experimental methods have recently been developed to detect allosteric binders that do not rely on the availability of a labeled probe. One such method is biointeraction chromatography, which quantifies the elution rate of a ligand from a protein immobilized in affinity chromatography columns in the absence or presence of another ligand (66). In this experimental setup, coadministration of an allosteric modulator results in a conformational change that alters the retention time of the probe ligand, thus changing its elution rate. Compared to orthosteric radioligand-based analyses, an advantage of biointeraction chromatography is that it can detect effects of modulators on low-affinity orthosteric ligands, whereas the binding assay is limited to the use of high-affinity probes. Another approach for detecting allosteric binders is affinity-selection mass spectrometry. This sensitive process uses chromatography followed by mass spectrometry to directly isolate ligand-protein complexes from a pool of complex mixtures of reactants. As with biointeraction chromatography, the presence of allosteric modulators in the reaction mixture changes the recovery of ligand samples in the chromatographic step, thus indicating a conformational change in the protein of interest (67).

With respect to GPCRs, there have been a number of elegant developments in spectroscopic and biophysical methods for monitoring receptor conformational changes. To date, these methods have generally been used to differentiate GPCR ligands on the basis of their efficacy; it remains to be determined if such methods have the capacity to differentiate them as orthosteric or allosteric ligands. Another novel approach applied to GPCR allosteric modulators, developed by Mandell and colleagues, utilizes a variation of signal analysis to computationally characterize patterns in GPCR structure and then use this information to design peptides that selectively target a given GPCR. This approach has led to the discovery of allosteric modulator peptides of D_2 dopamine and M_1 mAch receptors (68, 69).

WHERE ARE THE ALLOSTERIC SITES?

In contrast to many other proteins, GPCR structural studies have proceeded largely in the absence of a high-resolution X-ray crystal structure. The publication of the inactive-state bovine rhodopsin structure (63) has certainly helped, but has had only limited application thus far to the study of allosteric binding sites. Moreover, as mentioned in the Introduction, above, GPCRs display enormous diversity in the type of endogenous ligands they recognize; thus the domains that comprise the orthosteric site for one type of GPCR may represent an allosteric site for another. For example, the orthosteric site for many Family A GPCRs comprises regions in the transmembrane (TM) domain core, whereas for Family C receptors the orthosteric site is in the extracellular N terminus (6); for these latter receptors, allosteric modulators bind in the TM regions (see below).

Family A GPCRs

The Family A (rhodopsin-like) receptors represent the largest subfamily of GPCRs. Within this family, the most extensive structural studies of allosteric binding sites have been on mAch receptors. In part, this reflects the fact that the earliest evidence of allosteric modulation was noted for this receptor family (see 6, 70). It also reflects the utility of having a series of structurally diverse allosteric modulators that recognize at least one common allosteric site, albeit with different affinities, on all five subtypes of mAch receptors. Two basic approaches generally have been used in most mutagenesis studies of mAch receptor allosterism. The first has involved site-directed mutagenesis of residues that are conserved across each of the five subtypes of receptors, aiming to exploit commonalities within the allosteric domains. The second widespread approach has involved creation of chimeric receptors and mutagenesis of nonconserved residues in an effort to delineate the structural basis of modulator selectivity. Due to space constraints, an exhaustive overview is not presented. Interested readers are directed to other reviews (6, 70).

Using the M_2 mAch receptor as the prototypical model, most structure-function studies to date have localized the common allosteric site to domains comprising the second (E2) and third (E3) extracellular loops, as well as the top of TM domain 7. Thus, the highly acidic ^{172}EDGE^{175} sequence, specific to the E2 loop of the M_2 receptor, has long been postulated to contribute to the binding of positively charged modulators, especially gallamine (71, 72). Additional studies have identified Y^{177} in the E2 loop, N^{419} (and the corresponding K^{523} in the M_3 receptor) at the junction of E3/TM7, and T^{423} near the top of TM7 as playing crucial roles (2, 73–76). Of the conserved residues, W^{422} (corresponding to W^{401} in the M_1 receptor) in TM7 has also been implicated in the actions of allosteric modulators of mAch receptors (77, 78). Thus, in contrast to the orthosteric site, which is believed to comprise residues in the top third of the TM bundle core, the common allosteric site of the mAch receptors involves distinct residues that primarily utilize extracellular loop regions. Importantly, most mutations in these regions that affect allosteric modulator binding have minimal effects on binding of orthosteric ligands, further suggesting that the two types of ligands interact with topographically distinct domains.

Chemokine receptors are other Family A GPCR members that can be allosterically modulated by small-molecule inhibitors. Much focus has been on the CCR5 and CXCR4 subtypes of chemokine receptors owing to their relevance as targets in the treatment of HIV infection. Alanine scanning mutagenesis and molecular modeling of the TM helices of CCR5 have shown that the small molecule inhibitors TAK-779 and SCH-C bind near the extracellular surface of the receptor within a cavity formed between TM1, TM2, TM3, and TM7 (79, 80). A more detailed mutagenesis study has revealed that substitution of a number of residues in TM1 (L^{33} and Y^{37}), TM2 (V^{83}, W^{86} and A^{90}), TM5 (I^{198}), and TM7 (E^{283} and G^{286}) reduces the activity of three CCR5 inhibitors, TAK-779, SCH-C, and AD101. Additional mutational analysis has shown that three residues are required for the efficient interaction of all three compounds with CCR5: A negative charge in TM7 (E^{283}) is required for interaction with a positively charged nitrogen atom common to all three inhibitors, an interaction that also takes place between GSK873140 and CCR5 and between antagonists of other chemokine receptors (81). The aromatic moiety of Y^{37} in TM1 interacts with AD101 and SCH-C, while this residue's phenolic hydroxyl group is important for its interaction with TAK-779 (82). A hydrophobic aromatic group in TM2 (W^{86}) is also essential for interaction with each of these compounds. However, substitutions of other residues in the TM regions of CCR5 have more compound-specific effects (82). In addition, although mutagenesis has strongly suggested that TAK-779, SCH-C, and AD101 do not interact with the extracellular domains of CCR5, an interaction between GSK873140 and residues located in the E2 loop (R^{168}, C^{178}, and S^{180}) has been implicated in the binding of this compound to CCR5 (81, 83), suggesting subtle differences in the interaction of inhibitors with CCR5.

The first potent inhibitor of HIV interaction with CXCR4 was the bicyclam JM1657, later developed into JM3100 (or AMD3100) (84). The binding of AMD3100 has been studied using techniques similar to those employed to determine the small-molecule binding site of CCR5. Two negatively charged aspartate residues (D^{171} and D^{262}) located respectively in TM4 and TM6 of CXCR4 are particularly important for interaction with positively charged bicyclam molecules (85, 86). A glu residue in TM7 (E^{288}) provides an additional negative charge for the interaction of AMD3100 with CXCR4 (87). AMD3100 may also interact with several asp and phe residues (D^{181}, D^{182}, D^{187}, D^{193}, F^{199}, and F^{201}) in the E2 loop of CXCR4 (88).

More limited studies have assessed the location of the allosteric binding site on adenosine receptors. Kourounakis et al. (89) reported that mutation of T^{277} to alanine resulted in a decrease in both agonist affinity and the effects of the prototypical adenosine A_1 receptor allosteric modulator, PD 81723. T^{277} may thus be important in the conformational change involved in receptor activation and is consistent with a mechanism whereby the A_1 modulator, akin to orthosteric agonists, can promote receptor-G protein coupling. Gao et al. (90) investigated the residues involved in the allosteric modulation of the adenosine A_3 receptor, and identified N^{30}, D^{58}, D^{107}, F^{182}, or N^{274} as playing differential roles in the ability of various modulators to interact with that receptor.

Family B GPCRs

The most characterized Family B GPCRs are those that bind peptide hormones such as secretin, calcitonin, glucagon, and glucagon-like peptides (GLPs). These peptides range in length from ~27 to 52 amino acids and orthosteric binding is complex, involving interactions both with the large N-terminal extracellular domain of the receptor as well as the exoloops (91). Identification of small-molecule, nonpeptide drugs acting at Family B GPCRs has been problematic and, of those available, the mechanistic basis for their action is not well studied, but in many

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

cases is likely to be allosteric. Among the best characterized are antagonists of the corticotrophin-releasing factor (CRF) 1 receptor. These appear to bind within the TM domains of the receptor at sites distinct from those involved in peptide binding. For example, Liaw et al. (92) demonstrated that H^{199} (TM3) and M^{276} (TM5) are important for subtype selectivity of the compound NBI 27914 for CRF_1 over CRF_2 receptors. Mutation of these amino acids, however, did not affect peptide binding, consistent with the drug binding to a site distinct from that of the natural peptide ligand. Furthermore, the related compound, NBI 35965, slowed the dissociation of bound peptide ligands, indicative of allosteric modulation of orthosteric binding, an effect that may depend on the activation state of the receptor (93). Similarly, the glucagon receptor antagonist, L-168049, interacts with F184 and Y239 within TM2 and TM3, respectively; mutation of these residues does not alter binding of glucagon (94).

An interesting paradigm that occurs for selected Family B receptors is their modulation through interaction with the receptor activity modifying protein (RAMP) family of proteins (95). For example, three distinct receptor phenotypes arise from heterodimerization of the calcitonin receptor-like GPCR (CLR) with three individual RAMPs. Selective, high-affinity CGRP1 receptor (the heterodimer of CLR and RAMP1) antagonists have been developed that act at the interface between the GPCR and RAMP components (95). A key amino acid for this interaction is W^{74} of RAMP1, in which mutation to ala or lys leads to a 100-fold decrease in affinity of the compound BIBN4096BS without altering CGRP binding or activity (96). These data suggest that BIBN4096BS and related compounds may act allosterically and that the interface between stable GPCR-accessory protein interfaces can also constitute allosteric binding pockets.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

Family C GPCRs

Family C GPCRs, which include the mGluRs, $GABA_B$, and Ca^{2+}-sensing receptors, are characterized by a large bilobate extracellular orthosteric ligand-binding domain, commonly referred to as a "Venus flytrap" domain (97). These receptors form constitutive dimers, with the $GABA_B$ receptor identified as the first obligatory heterodimeric GPCR, being composed of two subunits, $GABA_{B1}$ and $GABA_{B2}$. The mechanism of activation of this heterodimeric complex is particularly interesting in that GABA binds to only one of the subunits ($GABA_{B1}$) to mediate G protein activation via the other ($GABA_{B2}$) subunit (97). Whether this mechanism of activation occurs for other GPCRs remains to be determined.

Allosteric modulators of mGluRs offer therapeutic potential in the treatment of disorders such as epilepsy, anxiety, pain, cognitive dysfunction, schizophrenia, and drug abuse. The binding site of such allosteric modulators of mGluR1 and mGluR5 receptors involves residues located on TM3, TM5, TM6, and TM7. Binding of the mGluR1-selective negative allosteric modulator, CPCCOEt, depends on T^{815} and A^{818} at the extracellular surface of TM7 (20). Malherbe et al. (98) demonstrated that T^{815}, but not A^{818}, was also involved in the binding of the allosteric inhibitor EM-TBPC to rat mGlu1 receptors but additional residues have also been implicated, including V^{757} located in TM5 and W^{798}, F^{801}, and Y^{805} located in TM6. (98). Lavreysen et al. (99) provided strong evidence for a common binding site for allosteric inhibitors acting at mGluRs by demonstrating that binding of the allosteric inhibitor [^{3}H]R214127 could be completely antagonized by a number of allosteric modulators.

MPEP is another potent and selective allosteric inhibitor of mGluR5 receptors (100). Site-directed mutagenesis has identified that residues from TM3, P^{654} and $Y^{658,}$ from TM5, L^{743}; TM6, T^{780}, W^{784}, F^{787}, and Y^{791}; and from TM7, A^{809}, are important for its actions (98). Similar to the mechanism of inhibition caused by EM-TBPC and CPCCOEt at mGluR1 receptors, MPEP has been proposed to interact with a cluster of aromatic residues on mGluR5 and impede the movement of TM6, thereby stabilizing the inactive form of the receptor (98). Pagano et al. (100) reported that binding of MPEP and its close analog, [^{3}H]M-MPEP, at human mGluR5 receptors involved I^{651}, P^{655}, and S^{658} in TM3, as well as A^{810} in TM7. By using receptor mutants of mGluR1 and mGluR5, Pagano et al. (100) also demonstrated that CPCCOEt could completely inhibit [^{3}H]M-MPEP binding in a concentration-dependent manner, indicating that some allosteric inhibitors of mGluR1 and mGluR5 occupy overlapping positions within the TM domains.

Allosteric enhancers of mGluRs have also been identified. The residue V^{757}, located in TM5 of rat mGluR1, appears to be critical for the activity of different classes of mGluR1 enhancers (101, 102). Two additional residues, S^{668} and C^{671}, located in TM3 of rat mGluR1, also appear to be involved in the binding of some of these modulators (101). At the mGluR2 receptor, G^{689}, S^{688}, and N^{735} have all been implicated in allosteric enhancer binding (103, 105). A number of residues important in the activity of the allosteric enhancer DFB at rat mGluR5 receptors have been reported: S^{657} in TM3; L^{743} in TM5; T^{780}, W^{784}, and F^{787} in TM6; and M^{801} in TM7 (106).

Human Ca^{2+}-sensing receptors can be allosterically modulated by L-amino acids, small peptides, Calhex 231, and a number of structurally related phenylalkylamine compounds such as NPS R-467, NPS R-568, and NPS 2143 (107–112). For example, the binding site of Ca^{2+} receptor allosteric inhibitors involves residues located in TM2, TM3, TM6, and TM7. NPS R-568 activity was reduced in Ca^{2+}-sensing receptors with mutations at F^{668} in TM2, F^{684} in TM3, or E^{837} at TM7 (111, 112). Residues F^{668}, R^{680}, F^{684}, and E^{837} have been implicated in NPS 2143 binding while F^{684}, F^{668}, W^{818}, E^{837}, and I^{841} have been implicated in Calhex 231 binding (110, 111).

Urwyler et al. (113) have identified two compounds, CGP7930 and CGP13501, that act as allosteric enhancers at the $GABA_B$ receptor. Binet et al. (31) demonstrated that CGP7930 activates a mutant receptor containing only the heptahelical domain of the $GABA_{B2}$ receptor but whether CGP7930 also binds to the heptahelical domain of the $GABA_{B1}$ receptor is not known. Given that GABA binds in the $GABA_{B1}$ subunit of the heterodimer, this latter finding suggests the intriguing possibility that allosteric modulation occurs across a dimer (see below).

The binding site of Family C GPCR allosteric modulators has thus far been demonstrated to reside predominantly within the TM domain of these receptors.

Interestingly, a number of residues that are critical for the binding of allosteric modulators at mGluRs and the Ca^{2+}-sensing receptor are overlapping. This suggests that although these compounds are often highly selective, they occupy a similar location within the TM domains.

Allosterism Across Dimers?

Given the evidence in recent years that GPCRs can form dimers or higher order oligomers, a logical question is whether allosteric interactions arise across such oligomeric arrays, and whether the allosteric site on a GPCR represents another conformationally linked orthosteric site in a dimer (or oligomer). As mentioned above, the interaction between the modulator, CGP7930, and GABA on the $GABA_B$ heterodimeric GPCR likely occurs across the dimer. However, the topography of the binding site for the modulator (TM region) is distinct from that of GABA (N-terminal domain). In contrast, mutational mapping of the binding site of Zn^{2+}, an allosteric agonist of the MC1 receptor, has identified residues on the inner face of TM3, which also contribute to the binding of the orthosteric agonist, α-MSH (36). This latter finding can be reconciled if the interaction is assumed to occur between two orthosteric sites that are allosterically linked in a receptor dimer.

Surprisingly, there have been very few adequately controlled studies that have investigated if cooperative binding is a general phenomenon for GPCRs. Recently, however, accumulating data suggest that this may be the case. For example, Mesnier & Banères (114) have used an elegant spectroscopic approach to demonstrate conformational changes in one monomer of the leukotriene B4 receptor by agonist occupancy of a second monomer linked to the first in a dimeric arrangement. Similarly, Urizar et al. (115) have provided kinetic evidence of negative cooperativity in the binding of orthosteric ligands to glycoprotein hormone receptors, and Springael et al. (116) have shown similar evidence for negative cooperativity between orthosteric ligands that bind to chemokine receptor homo- and heterooligomers. Indeed, if all GPCRs function as obligate oligomers, binding cooperativity should be readily demonstrable.

In general, there is good evidence for many allosteric sites on GPCRs being topographically distinct from the orthosteric site (see above). When considering whether mutagenesis data that suggest a close proximity of attachment points for orthosteric and allosteric ligands implies that the sites are identical, it is informative to examine findings with enzymes for which crystal structures have been solved with allosteric modulators bound to the protein. In the case of p38 MAP kinase, for example, Pargellis et al. (117) reported that the allosteric inhibitor, BIRB-796, bound in a pocket that did not utilize atoms that contributed to the binding of ATP to the enzyme's active site. Nonetheless, the crystal structure of the complex showed that the BIRB-796 pocket was immediately adjacent to the ATP binding pocket, highlighting the fact that allosteric sites can be very close to but topographically distinct from orthosteric sites. Interestingly, a recent study by Trankle et al. (118) has shown that two molecules of tacrine, an allosteric modulator of mAch receptors, can bind cooperatively within the common allosteric site on a single M_2 mAch receptor monomer. Overall, however, it remains to be established whether interactions between distinct orthosteric and allosteric sites on GPCRs imply interaction within a monomer or across a dimer. It is likely that both situations will be shown to exist.

ENDOGENOUS ALLOSTERIC MODULATORS

Although the preceding sections have focused on exogenous allosteric modulators, evidence exists for endogenous modulators of various GPCRs. The G protein itself, as well as the expanding list of GPCR-interacting proteins, are examples of endogenous allosteric GPCR-protein partners. Similarly, if GPCRs function as dimers or higher order oligomers, then every endogenous orthosteric ligand for a given receptor is also a potential allosteric modulator as a consequence of cooperativity. However, evidence exists for other endogenous molecules, including ions, lipids, and peptides, acting as allosteric modulators for GPCRs.

The binding of ions such as Zn^{2+}, Na^+, and Ca^{2+} is important for the function of numerous ligands at different GPCRs. For example, Zn^{2+} can allosterically modulate the activity of orthosteric ligands at D_1, D_2 (119, 120), and D_4 (121) dopamine receptors; MC1 and MC4 melanocortin receptors (36); β_2 adrenergic (122); and μ opioid receptors (123). The putative binding site for Zn^{2+} differs among receptors, as do the manifestations of its allosteric effect. For example, for the MC1 melanocortin receptor, Zn^{2+} binding is believed to involve C^{271}, located in E3, and D^{119}, at the extracellular end of TM3 (36). At the β_2 adrenergic receptor, Zn^{2+} has been suggested to interact with the intracellular ends of TM5 (E^{255}) and TM6 (H^{269}; C^{265}) to enhance agonist affinity (122). Binding of Zn^{2+} to the μ opioid receptor involves TM7 (H^{319}) and E2 (D^{216}) and results in reduction of both agonist and antagonist binding (123). Zn^{2+} also reduces orthosteric antagonist binding at the D_2 and D_4 dopamine receptors, and studies on D_2 receptors have implicated two residues located toward the extracellular end of TM6 (H^{392} and H^{399}) in its allosteric actions (119).

Na^+ can modulate the ability of Zn^{2+} to inhibit antagonist binding to the D_4 dopamine receptor (121), indicating that two or more allosteric sites within a GPCR can be occupied simultaneously by ions to regulate the orthosteric site and each other. The ability of Na^+ to allosterically modulate the activity of orthosteric ligands at GPCRs has been well documented for more than two decades. Site-directed mutagenesis studies have indicated that a highly conserved aspartate in TM2 is important for mediating this effect in different GPCRs (6). The conservation of this site likely reflects its importance in receptor activation and the role of Na^+ in stabilization of the ground (inactive) GPCR state (124).

Ca^{2+} is another ion involved in GPCR allosterism: it can act both as an allosteric modulator of some GPCRs and as an orthosteric ligand that itself is subject to modulation. For example, the absence of Ca^{2+} can greatly reduce the affinity and potency of certain agonists and antagonists at the $GABA_B$ heterodimeric receptor, an effect dependent on S^{269} in the $GABA_BR1$ subunit (125). Conversely, aromatic and aliphatic L-amino acids can potentiate the actions of Ca^{2+} as the orthosteric agonist of the

Ca^{2+}-sensing receptor (126) by binding to a site in the receptor that is close to, but topographically distinct from, the orthosteric Ca^{2+} binding site.

A number of reports have suggested the existence of endogenous allosteric peptide modulators of GPCR activity. For instance, the small tetrapeptide, 5-HT-moduline (Leu-Ser-Ala-Leu/LSAL), which was originally isolated from rat brain, is an allosteric modulator highly specific for the 5-HT_{1B} receptor, where it acts as an insurmountable inhibitor of ligand binding (127). This effect yields inhibition of agonist action in terms of G protein coupling (128) and in a mouse model of social interaction (127). The tripeptide, Pro-Leu-Gly (PLG), also known as melanotropin release inhibiting factor-1 (MIF-1), is an endogenous allosteric modulator of D_2 and D_4 dopamine receptors, where it promotes a high-affinity receptor conformation and enhances the binding and activity of orthosteric agonists (129, 130). This effect is reminiscent of the action of allosteric enhancers of the A_1 adenosine receptor (see above).

MAch receptors may also be regulated by allosteric peptides, including the κ opioid receptor agonist, dynorphin, and polycationic peptides, such as poly-L-arginine, poly-L-lysine, and poly-L-ornithine (6). Interestingly, human eosinophil major basic protein and eosinophil peroxidase may act as allosteric antagonists of the M_2 mAch receptor (6). This finding is of potential pathophysiological relevance because antagonism of the M_2 receptors may be responsible for some of the processes associated with diseases of the airways: decreased binding of acetylcholine to neuronal autoinhibitory M_2 mAch receptors can lead to increased bronchoconstriction (131).

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

Endogenous lipids can also allosterically regulate GPCRs. For instance, the amidated lipid, oleamide, regulates the activity of 5-HT at several 5-HT receptor subtypes, including the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7 receptors (132, 133). The endocannabinoid receptor agonist, anandamide, can also modify the binding and function of ligands at M_1 and M_4 mAch receptors in a noncompetitive manner (55). The steroid hormone, progesterone, inhibits the maximum binding of oxytocin and arginine vasopressin to the rat oxytocin receptor and the progesterone metabolite, 5β-dihydroprogesterone, alters the binding of oxytocin to the human oxytocin receptor (134). Cholesterol can similarly regulate ligand binding to the oxytocin receptor. Removal of cholesterol from cell membranes decreases the maximum binding of oxytocin, whereas the presence of cholesterol stabilizes the receptor in a high-affinity conformation and decreases dissociation of oxytocin (135). Modifications in receptor function may result from an alteration in membrane fluidity or membrane microdomains following changes in the membrane sterol content. However, molecular modeling has suggested that cholesterol interacts with nonpolar residues located in TM5 (P^{197}, Y^{200}, and W^{203}) and TM6 (M^{296} and W^{297}) in the oxytocin receptor (136), in contrast to the putative oxytocin binding site that is believed to be located in the extracellular domains of the receptor.

There are a number of diseases that appear to be mediated, at least in part, by the actions of endogenous GPCR modulators. For example, Chagas' disease, an infection caused by the parasite *Trypanosoma cruzi*, involves autoantibodies that bind to the M_2 mAch and β_1 adrenergic receptors and cause insurmountable inhibition of the binding of receptor ligands through an interaction with the second extracellular loop of the receptors (137, 140). The antibodies additionally stimulate agonist-like receptor activity, which leads to a decrease and increase in atrial contraction through the mAch and adrenergic receptors, respectively (137, 139). It is believed that this chronic receptor activation leads to damage to cardiac tissue in this disease.

Sjögren's syndrome is another autoimmune disease in which autoantibodies appear to activate mAch receptors (141), although some confusion exists regarding the epitope and the receptor subtype that these antibodies target. Nonetheless, the recognition that certain disorders are characterized by the presence of allosteric autoantibodies suggests a role for neutrally cooperative ligands ($\alpha = 1$ with respect to the endogenous transmitter) as potential therapeutics; theoretically, these ligands could block the action of the allosteric autoantibodies but not those of endogenous orthosteric agonists.

As described in the preceding section, the extracellular loop regions are important for the binding of different allosteric modulators to some GPCRs. Interestingly, autoantibodies from schizophrenic patients have also shown reactivity against the E2 loop of the M_1 and M_2 mAch receptors, where they stimulate receptor signaling (142). Similarly, antibodies present in patients with preeclamsia can activate the AT_1 angiotensin receptor; this activation is inhibited by a synthetic peptide corresponding to a portion of the E2 loop of the receptor (143). Synthetic antibodies raised against E2 in the M_2 mAch and β_1 adrenergic receptors also have agonist effects (144, 145), whereas single-chain variable fragments can possess inverse agonist activity at M_2 mAch and β_2 adrenergic receptors (146, 147), suggesting that E2 may play an important function for these GPCRs. Antibodies against E2 in the 5-HT_{4e} receptor also possess agonist-like activity (148), whereas those against the 5-HT_{1A} serotonin receptor can stabilize the high-affinity agonist-bound receptor conformation (149), suggesting a common allosteric binding site for antibodies possessing activity at GPCRs.

The above findings imply that endogenous allosteric modulators of GPCR activity may play important roles in physiological and pathophysiological processes. Although every GPCR will not necessarily be associated with unique allosteric sites for endogenous modulators, the list of such endogenous substances is likely to increase.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

CONCLUSIONS

Allosteric modulators tremendously expand the chemical space of potential GPCR therapeutic agents, and many of the theoretical behaviors associated with such compounds are being rapidly identified. The great challenges facing the field are the optimization of screening assays for the reliable detection/validation of allosteric modulator effects and the further development of novel methodologies for studying protein allostery, including a better structural identification of allosteric binding sites. Given the preeminence of GPCRs as therapeutic targets, the rewards of successfully meeting these challenges are significant.

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

SUMMARY POINTS

1. Many GPCRs contain allosteric binding sites, which are topographically distinct from the orthosteric site recognized by the endogenous agonist.
2. Binding to an allosteric site on a GPCR changes receptor conformation and can modulate the binding affinity of orthosteric ligands, the signaling efficiency of orthosteric ligands, or can perturb signaling even in the absence of orthosteric ligand.
3. Allosteric sites represent attractive drug targets for small molecules because they can engender selectivity across receptor subtypes through binding to nonconserved regions and/or cooperativity with orthosteric ligands at a particular receptor subtype. Moreover, allosteric modulators that exert their effects only in the presence of the endogenous agonist may provide a safer pharmacological profile than do orthosteric agonists and antagonists.
4. A great challenge for GPCR-based drug discovery is the ability to reliably detect, validate, and quantify the actions of allosteric modulators. This challenge results because allosteric effects can vary depending on the nature of the orthosteric ligand and are limited by the degree of cooperativity between allosteric and orthosteric sites.
5. Structural studies are beginning to elucidate the molecular determinants of allosteric sites on GPCRs. Domains that contribute to the orthosteric pocket on one type of GPCR can contribute to an allosteric pocket for another GPCR.
6. Although allosteric sites need not have evolved to accommodate endogenous ligands, ions, lipids, and peptides can serve as endogenous allosteric modulators for some GPCRs.

DISCLOSURE STATEMENT

A.C. and P.S. received funding support from GlaxoSmithKline and Pfizer for collaborative research on allosteric modulators and funding support from the National Health and Medical Research Council of Australia for the study of allosteric modulation of GPCRs. A.C. is a consultant for Addex Pharmaceuticals on screening for allosteric modulators.

LITERATURE CITED

1. Foord S. 2002. Receptor classification: post genome. *Curr. Opin. Pharmacol.* 2:561
2. **Christopoulos A. 2002. Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat. Rev. Drug Discov.* 1:198–210**
3. Bouvier M. 2001. Oligomerization of G-protein-coupled transmitter receptors. *Nat. Rev. Neurosci.* 2:274–86

2. Concise overview of allosterism at cell-surface receptors.

4. Brady AE, Limbird LE. 2002. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell Signal* 14:297–309
5. Bohr C, Hasselbalch KA, Krogh A. 1904. Ubereinen in biologischen Beziehung wichtigen Einfluss, den die Kohlen-sauerspannung des Blutes auf dessen Sauerstoffbindung ubt. *Skand. Arch. Physiol.* 16:402–12

6. Comprehensive overview of the development of concepts, molecular mechanisms, and issues related to screening for allosteric modulators of GPCRs.

6. **Christopoulos A, Kenakin T. 2002. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* 54:323–74**
7. Wyman J, Allen DW. 1951. The problem of heme interactions in hemoglobin and the basis for the Bohr effect. *J. Polymer. Sci.* 7:499–518
8. Katz B, Thesleff S. 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* 138:63–80

9 and 10. The classic papers introducing the concept of allsoterism.

9. **Monod J, Changeux JP, Jacob F. 1963. Allosteric proteins and cellular control systems. *J. Mol. Biol.* 6:306–29**
10. **Monod J, Wyman J, Changeux JP. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12:88–118**
11. Ehlert FJ. 1985. The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol. Pharmacol.* 28:410–21
12. Neubig RR, Spedding M, Kenakin T, Christopoulos A. 2003. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* 55:597–606
13. Kenakin T. 2005. New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nat. Rev. Drug Discov.* 4:919–27
14. Lockless SW, Ranganathan R. 1999. Evolutionarily conserved pathways of energetic connectivity in protein families. *Science* 286:295–99
15. Hardy JA, Wells JA. 2004. Searching for new allosteric sites in enzymes. *Curr. Opin. Struct. Biol.* 14:706–15
16. Swain JF, Gierasch LM. 2006. The changing landscape of protein allostery. *Curr. Opin. Struct. Biol.* 16:102–8
17. Freire E. 2000. Can allosteric regulation be predicted from structure? *Proc. Natl. Acad. Sci. USA* 97:11680–82

18. An important paper outlining the properties and application of the allosteric ternary complex model.

18. **Ehlert FJ. 1988. Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol. Pharmacol.* 33:187–94**
19. Kenakin TP. 1997. *Pharmacologic Analysis of Drug-Receptor Interaction*. Philadelphia, PA: Lippincott-Raven
20. Litschig S, Gasparini F, Rueegg D, Stoehr N, Flor PJ, et al. 1999. CPCCOEt, a noncompetitive metabotropic glutamate receptor 1 antagonist, inhibits receptor signaling without affecting glutamate binding. *Mol. Pharmacol.* 55:453–61
21. Urwyler S, Mosbacher J, Lingenhoehl K, Heid J, Hofstetter K, et al. 2001. Positive allosteric modulation of native and recombinant gamma-aminobutyric acid(B) receptors by 2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its aldehyde analog CGP13501. *Mol. Pharmacol.* 60:963–71

22. Zahn K, Eckstein N, Trankle C, Sadee W, Mohr K. 2002. Allosteric modulation of muscarinic receptor signaling: alcuronium-induced conversion of pilocarpine from an agonist into an antagonist. *J. Pharmacol. Exp. Ther.* 301:720–28

23. Watson C, Jenkinson S, Kazmierski W, Kenakin T. 2005. The CCR5 receptor-based mechanism of action of 873140, a potent allosteric noncompetitive HIV entry inhibitor. *Mol. Pharmacol.* 67:1268–82

24. Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, et al. 2005. Allosteric modulation of the cannabinoid CB1 receptor. *Mol. Pharmacol.* 68:1484–95

25. Ehlert FJ. 2005. Analysis of allosterism in functional assays. *J. Pharmacol. Exp. Ther.* 315:740–54

26. Kenakin T. 2004. Allosteric modulators: the new generation of receptor antagonist. *Mol. Interv.* 4:222–29

27. Hall DA. 2000. Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. *Mol. Pharmacol.* 58:1412–23

28. Bruns RF, Fergus JH. 1990. Allosteric enhancement of adenosine A1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol. Pharmacol.* 38:939–49

29. Figler H, Olsson RA, Linden J. 2003. Allosteric enhancers of A1 adenosine receptors increase receptor-G protein coupling and counteract Guanine nucleotide effects on agonist binding. *Mol. Pharmacol.* 64:1557–64

30. Sachpatzidis A, Benton BK, Manfredi JP, Wang H, Hamilton A, et al. 2003. Identification of allosteric peptide agonists of CXCR4. *J. Biol. Chem.* 278:896–907

31. Binet V, Brajon C, Le Corre L, Acher F, Pin JP, Prezeau L. 2004. The heptahelical domain of GABA(B2) is activated directly by CGP7930, a positive allosteric modulator of the GABA(B) receptor. *J. Biol. Chem.* 279:29085–91

32. Goudet C, Gaven F, Kniazeff J, Vol C, Liu J, et al. 2004. Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors. *Proc. Natl. Acad. Sci. USA* 101:378–83

33. Mitsukawa K, Yamamoto R, Ofner S, Nozulak J, Pescott O, et al. 2005. A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. *Proc. Natl. Acad. Sci. USA* 102:18712–17

34. Holst B, Brandt E, Bach A, Heding A, Schwartz TW. 2005. Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling. *Mol. Endocrinol.* 19:2400–11

35. Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, et al. 2006. Probing the molecular mechanism of interaction between 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M(1) receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. *Mol. Pharmacol.* 69:236–46

24. Striking illustration of the dissimilitude of effects of allosteric modulators on binding versus signaling.

27. Development of a molecular model describing allosteric effects on affinity and efficacy.

36. Holst B, Elling CE, Schwartz TW. 2002. Metal ion-mediated agonism and agonist enhancement in melanocortin MC1 and MC4 receptors. *J. Biol. Chem.* 277:47662–70

37. Kinney GG, O'Brien JA, Lemaire W, Burno M, Bickel DJ, et al. 2005. A novel selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 has in vivo activity and antipsychotic-like effects in rat behavioural models. *J. Pharmacol. Exp. Ther.* 313:199–206

38. Schwartz TW, Holst B. 2006. Ago-allosteric modulation and other types of allostery in dimeric 7TM receptors. *J. Recept. Signal Transduct. Res.* 26:107–28

39. Hoare SRJ, Strange PG. 1996. Regulation of D_2 dopamine receptors by amiloride and amiloride analogs. *Mol. Pharmacol.* 50:1295–308

40. Lazareno S, Popham A, Birdsall NJ. 2000. Allosteric interactions of staurosporine and other indolocarbazoles with N-[methyl-^{3}H]scopolamine and acetylcholine at muscarinic receptor subtypes: identification of a second allosteric site. *Mol. Pharmacol.* 58:194–207

41. Durroux T. 2005. Principles: a model for the allosteric interactions between ligand binding sites within a dimeric GPCR. *Trends Pharmacol. Sci.* 26:376–84

42. Christopoulos A, Parsons AM, Lew MJ, El-Fakahany EE. 1999. The assessment of antagonist potency under conditions of transient response kinetics. *Eur. J. Pharmacol.* 382:217–27

43. Gao ZG, Van Muijlwijk-Koezen JE, Chen A, Muller CE, Ijzerman AP, Jacobson KA. 2001. Allosteric modulation of a(3) adenosine receptors by a series of 3-(2-pyridinyl)isoquinoline derivatives. *Mol. Pharmacol.* 60:1057–63

44. Lazareno S, Dolezal V, Popham A, Birdsall NJM. 2004. Thiochrome enhances acetylcholine affinity at muscarinic M_4 receptors: receptor subtype selectivity via cooperativity rather than affinity. *Mol. Pharmacol.* 65:257–66

45. Portoghese PS. 1989. Bivalent ligands and the message-address concept in the design of selective opioid receptor antagonists. *Trends Pharmacol. Sci.* 10:230–35

46. Disingrini T, Muth M, Dallanoce C, Barocelli E, Bertoni S, et al. 2006. Design, synthesis, and action of oxotremorine-related hybrid-type allosteric modulators of muscarinic acetylcholine receptors. *J. Med. Chem.* 49:366–72

47. May LT, Avlani VA, Sexton PM, Christopoulos A. 2004. Allosteric modulation of G protein-coupled receptors. *Curr. Pharm. Des.* 10:2003–13

48. Klaasse EC, van den Hout G, Roerink SF, de Grip WJ, Ijzerman AP, Beukers MW. 2005. Allosteric modulators affect the internalization of human adenosine A(1) receptors. *Eur. J. Pharmacol.* 522:1–8

49. May LT, Lin Y, Sexton PM, Christopoulos A. 2005. Regulation of M2 muscarinic acetylcholine receptor expression and signaling by prolonged exposure to allosteric modulators. *J. Pharmacol. Exp. Ther.* 312:382–90

50. Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, et al. 2002. Discovery of an ectopic activation site on the M(1) muscarinic receptor. *Mol. Pharmacol.* 61:1297–302

51. Motulsky HJ, Mahan LC. 1984. The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol. Pharmacol.* 25:1–9

52. **Lazareno S, Birdsall NJM. 1995. Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol.* 48:362–78**
53. Kostenis E, Mohr K. 1996. Composite action of allosteric modulators on ligand binding. *Trends Pharmacol. Sci.* 17:443–44
54. Avlani VA, May LT, Sexton PM, Christopoulos A. 2004. Application of a kinetic model to the apparently complex behaviors of negative and positive allosteric modulators of muscarinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 308:1062–72
55. Lanzafame AA, Guida E, Christopoulos A. 2004. Effects of anandamide on the binding and signaling properties of M1 muscarinic acetylcholine receptors. *Biochem. Pharmacol.* 68:2207–19
56. Lanzafame A, Christopoulos A. 2004. Investigation of the interaction of a putative allosteric modulator, SCH-202676, with M_1 muscarinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.* 308:830–37
57. Lazareno S, Popham A, Birdsall NJ. 2002. Analogs of WIN 62,577 define a second allosteric site on muscarinic receptors. *Mol. Pharmacol.* 62:1492–505
58. Ellis J, Seidenberg M. 1992. Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol. Pharmacol.* 42:638–41
59. Rees S, Morrow D, Kenakin T. 2002. GPCR drug discovery through the exploitation of allosteric drug binding sites. *Receptors Channels* 8:261–68
60. Christopoulos A, Mitchelson F. 1994. Assessment of the allosteric interactions of the bisquaternary heptane-1,7-bis(dimethyl-3′-pthalimidopropyl)ammonium bromide at M_1 and M_2 muscarine receptors. *Mol. Pharmacol.* 46:105–14
61. Christopoulos A, Mitchelson F. 1997. Application of an allosteric ternary complex model to the technique of pharmacological resultant analysis. *J. Pharm. Pharmacol.* 49:781–86
62. Urwyler S, Pozza MF, Lingenhoehl K, Mosbacher J, Lampert C, et al. 2003. N,N′-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of gamma-aminobutyric acidB receptor function. *J. Pharmacol. Exp. Ther.* 307:322–30
63. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, et al. 2000. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289:739–45
64. Cavasotto CN, Orry AJ, Abagyan RA. 2003. Structure-based identification of binding sites, native ligands and potential inhibitors for G-protein coupled receptors. *Proteins* 51:423–33
65. An J, Totrov M, Abagyan R. 2005. Pocketome via comprehensive identification and classification of ligand binding envelopes. *Mol. Cell Proteomics* 4:752–61
66. Chen J, Hage DS. 2004. Quantitative analysis of allosteric drug-protein binding by biointeraction chromatography. *Nat. Biotechnol.* 22:1445–48

52. Comprehensive description of approaches to detecting and validating allosteric effects at GPCRs.

67. Annis DA, Nazef N, Chuang CC, Scott MP, Nash HM. 2004. A general technique to rank protein-ligand binding affinities and determine allosteric versus direct binding site competition in compound mixtures. *Am. Chem. Soc.* 126:15495–503
68. Mandell AJ, Selz KA, Owens MJ, Kinkead B, Shlesinger MF, et al. 2003. Cellular and behavioural effects of D2 dopamine receptor hydrophobic eigenmode-targeted peptide ligands. *Neuropsychopharmacology* 28:S98–107
69. Selz KA, Mandell AJ, Shlesinger MF, Arcuragi V, Owens MJ. 2004. Designing human M1 muscarinic receptor-targeted hydrophobic eigenmode matched peptides as functional modulators. *Biophys. J.* 86:1308–31
70. Christopoulos A, Lanzafame A, Mitchelson F. 1998. Allosteric interactions at muscarinic cholinoceptors. *Clin. Exp. Pharmacol. Physiol.* 25:184–94
71. Leppik RA, Miller RC, Eck M, Paquet JL. 1994. Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the M2 muscarinic acetylcholine receptor. *Mol. Pharmacol.* 45:983–90
72. Gnagey AL, Seidenberg M, Ellis J. 1999. Site-directed mutagenesis reveals two epitopes involved in the subtype selectivity of the allosteric interactions of gallamine at muscarinic acetylcholine receptors. *Mol. Pharmacol.* 56:1245–53
73. Krejci A, Tucek S. 2001. Changes of cooperativity between N-methylscopolamine and allosteric modulators alcuronium and gallamine induced by mutations of external loops of muscarinic M(3) receptors. *Mol. Pharmacol.* 60:761–67
74. Buller S, Zlotos DP, Mohr K, Ellis J. 2002. Allosteric site on muscarinic acetylcholine receptors: a single amino acid in transmembrane region 7 is critical to the subtype selectivities of caracurine V derivatives and alkane-bisammonium ligands. *Mol. Pharmacol.* 61:160–68
75. Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, et al. 2003. Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M2 receptor that account entirely for the M2/M5 subtype selectivities of some structurally diverse allosteric ligands in N-methylscopolamine-occupied receptors. *Mol. Pharmacol.* 64:21–31
76. Huang XP, Prilla S, Mohr K, Ellis J. 2005. Critical amino acid residues of the common allosteric site on the M2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide. *Mol. Pharmacol.* 68:769–78
77. Matsui H, Lazareno S, Birdsall NJ. 1995. Probing of the location of the allosteric site on M1 muscarinic receptors by site-directed mutagenesis. *Mol. Pharmacol.* 47:88–98
78. Prilla S, Schrobang J, Ellis J, Holtje HD, Mohr K. 2006. Allosteric interactions with muscarinic acetylcholine receptors: complex role of the conserved tryptophan M2422Trp in a critical cluster of amino acids for baseline affinity, subtype selectivity, and cooperativity. *Mol. Pharmacol.* 70:181–93
79. Dragic T, Trkola A, Thompson DA, Cormier EG, Kajumo FA, et al. 2000. A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc. Natl. Acad. Sci. USA* 97:5639–44

80. Tsamis F, Gavrilov S, Kajumo F, Seibert C, Kuhmann S, et al. 2003. Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J. Virol.* 77:5201–8
81. Maeda K, Das D, Ogata-Aoki H, Nakata H, Miyakawa T, et al. 2006. Structural and molecular interactions of CCR5 inhibitors with CCR5. *J. Biol. Chem.* 281(18):12688–98
82. Seibert C, Ying W, Gavrilov S, Tsamis F, Kuhmann SE, et al. 2006. Interaction of small molecule inhibitors of HIV-1 entry with CCR5. *Virology* 349(1):41–54
83. Maeda K, Nakata H, Koh Y, Miyakawa T, Ogata H, et al. 2004. Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro. *J. Virol* 78:8654–62
84. De Clercq E. 2003. The bicyclam AMD3100 story. *Nat. Rev. Drug Discov.* 2:581–87
85. Gerlach LO, Skerlj RT, Bridger GJ, Schwartz TW. 2001. Molecular interactions of cyclam and bicyclam nonpeptide antagonists with the CXCR4 chemokine receptor. *J. Biol. Chem.* 276:14153–60
86. Hatse S, Princen K, Gerlach LO, Bridger G, Henson G, et al. 2001. Mutation of Asp(171) and Asp(262) of the chemokine receptor CXCR4 impairs its coreceptor function for human immunodeficiency virus-1 entry and abrogates the antagonistic activity of AMD3100. *Mol. Pharmacol.* 60:164–73
87. Rosenkilde MM, Gerlach LO, Jakobsen JS, Skerlj RT, Bridger GJ, Schwartz TW. 2004. Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. *J. Biol. Chem.* 279:3033–41
88. Labrosse B, Brelot A, Heveker N, Sol N, Schols D, et al. 1998. Determinants for sensitivity of human immunodeficiency virus coreceptor CXCR4 to the bicyclam AMD3100. *J. Virol* 72:6381–88
89. Kourounakis A, Visser C, de Groote M, IJzerman AP. 2001. Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-trifluoromethyl) phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A1 receptor. *Biochem. Pharmacol.* 61:137–44
90. Gao ZG, Kim SK, Gross AS, Chen A, Blaustein JB, Jacobson KA. 2003. Identification of essential residues involved in the allosteric modulation of the human A(3) adenosine receptor. *Mol. Pharmacol.* 63:1021–31
91. Hoare SR. 2005. Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discov. Today* 10:417–27
92. Liaw CW, Grigoriadis DE, Lorang MT, De Souza EB, Maki RA. 1997. Localization of agonist- and antagonist-binding domains of human corticotropin-releasing factor receptors. *Mol. Endocrinol.* 11:2048–53
93. Hoare SR, Sullivan SK, Ling N, Crowe PD, Grigoriadis DE. 2003. Mechanism of corticotropin-releasing factor type I receptor regulation by nonpeptide antagonists. *Mol. Pharmacol.* 63:751–65

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

94. Cascieri MA, Koch GE, Ber E, Sadowski SJ, Louizides D, et al. 1999. Characterization of a novel, nonpeptidyl antagonist of the human glucagon receptor. *J. Biol. Chem.* 274:8694–97

95. Hay DL, Poyner DR, Sexton PM. 2006. GPCR modulation by RAMPs. *Pharmacol. Ther*. 109:173–97

96. Mallee JJ, Salvatore CA, LeBourdelles B, Oliver KR, Longmore J, et al. 2002. Receptor activity-modifying protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. *J. Biol. Chem.* 277:14294–98

97. Pin JP, Kniazeff J, Liu J, Binet V, Goudet C, et al. 2005. Allosteric functioning of dimeric class C G-protein-coupled receptors. *FEBS J.* 272:2947–55

98. Malherbe P, Kratochwil N, Knoflach F, Zenner MT, Kew JN, et al. 2003. Mutational analysis and molecular modeling of the allosteric binding site of a novel, selective, noncompetitive antagonist of the metabotropic glutamate 1 receptor. *J. Biol. Chem.* 278:8340–47

99. Lavreysen H, Janssen C, Bischoff F, Langlois X, Leysen JE, Lesage AS. 2003. [3H]R214127: a novel high-affinity radioligand for the mGlu1 receptor reveals a common binding site shared by multiple allosteric antagonists. *Mol. Pharmacol.* 63:1082–93

100. Pagano A, Ruegg D, Litschig S, Stoehr N, Stierlin C, et al. 2000. The noncompetitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J. Biol. Chem.* 275:33750–58

101. Knoflach F, Mutel V, Jolidon S, Kew JN, Malherbe P, et al. 2001. Positive allosteric modulators of metabotropic glutamate 1 receptor: characterization, mechanism of action, and binding site. *Proc. Natl. Acad. Sci. USA* 98:13402–7

102. Hemstapat K, de Paulis T, Chen Y, Brady AE, Grover VK, et al. 2006. A novel class of positive allosteric modulators of mGluR1 interact with a site distinct from that of negative allosteric modulators. *Mol. Pharmacol.* 70(2):616–26

103. Baez M, Yang P, Kandasamy R, Large T, Kelly G, et al. 2002. Molecular mapping of a subtype selective site for positive allosteric modulation of the mGlu2 receptor. *Neuropharmacology* 43:275

104. Galici R, Jones CK, Hemstapat K, Nong Y, Echemendia NG, et al. 2006. Biphenyl-indanone A, a positive allosteric modulator of the metabotropic glutamate receptor subtype 2, has antipsychotic- and anxiolytic-like effects in mice. *J. Pharmacol. Exp. Ther.* In press

105. Schaffhauser H, Rowe BA, Morales S, Chavez-Noriega LE, Yin R, et al. 2003. Pharmacological characterization and identification of amino acids involved in the positive modulation of metabotropic glutamate receptor subtype 2. *Mol. Pharmacol.* 64:798–810

106. Muhlemann A, Ward NA, Kratochwil N, Diener C, Fischer C, et al. 2006. Determination of key amino acids implicated in the actions of allosteric modulation by 3,3′-difluorobenzaldazine on rat mGlu5 receptors. *Eur. J. Pharmacol.* 529:95–104

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

107. Nemeth EF, Steffey ME, Hammerland LG, Hung BC, Van Wagenen BC, et al. 1998. Calcimimetics with potent and selective activity on the parathyroid calcium receptor. *Proc. Natl. Acad. Sci. USA* 95:4040–45
108. Nemeth EF, Delmar EG, Heaton WL, Miller MA, Lambert LD, et al. 2001. Calcilytic compounds: potent and selective Ca^{2+} receptor antagonists that stimulate secretion of parathyroid hormone. *J. Pharmacol. Exp. Ther.* 299:323–31
109. Hammerland LG, Garrett JE, Hung BC, Levinthal C, Nemeth EF. 1998. Allosteric activation of the Ca^{2+} receptor expressed in *Xenopus laevis* oocytes by NPS 467 or NPS 568. *Mol. Pharmacol.* 53:1083–88
110. Petrel C, Kessler A, Maslah F, Dauban P, Dodd RH, et al. 2003. Modeling and mutagenesis of the binding site of Calhex 231, a novel negative allosteric modulator of the extracellular Ca(2+)-sensing receptor. *J. Biol. Chem.* 278:49487–94
111. Miedlich SU, Gama L, Seuwen K, Wolf RM, Breitwieser GE. 2004. Homology modeling of the transmembrane domain of the human calcium sensing receptor and localization of an allosteric binding site. *J. Biol. Chem.* 279:7254–63
112. Hu J, Reyes-Cruz G, Chen W, Jacobson KA, Spiegel AM. 2002. Identification of acidic residues in the extracellular loops of the seven-transmembrane domain of the human Ca^{2+} receptor critical for response to Ca^{2+} and a positive allosteric modulator. *J. Biol. Chem.* 277:46622–31
113. Urwyler S, Gjoni T, Kaupmann K, Pozza MF, Mosbacher J. 2004. Selected amino acids, dipeptides and arylalkylamine derivatives do not act as allosteric modulators at GABAB receptors. *Eur. J. Pharmacol.* 483:147–53
114. Mesnier D, Baneres JL. 2004. Cooperative conformational changes in a G-protein-coupled receptor dimer, the leukotriene B(4) receptor BLT1. *J. Biol. Chem.* 279:49664–70
115. Urizar E, Montanelli L, Loy T, Bonomi M, Swillens S, et al. 2005. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J.* 24:1954–64
116. Springael JY, Le Minh PN, Urizar E, Costagliola S, Vassart G, Parmentier M. 2006. Allosteric modulation of binding properties between units of chemokine receptor homo- and hetero-oligomers. *Mol. Pharmacol.* 69:1652–61
117. Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, et al. 2002. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nat. Struct. Biol.* 9:268–72
118. Trankle C, Dittmann A, Schulz U, Weyand O, Buller S, et al. 2005. Atypical muscarinic allosteric modulation: cooperativity between modulators and their atypical binding topology in muscarinic M2 and M2/M5 chimeric receptors. *Mol. Pharmacol.* 68:1597–610
119. Liu Y, Teeter MM, DuRand CJ, Neve KA. 2006. Identification of a Zn^{2+}-binding site on the dopamine D2 receptor. *Biochem. Biophys. Res. Commun.* 339:873–79
120. Schetz JA, Sibley DR. 1997. Zinc allosterically modulates antagonist binding to cloned D1 and D2 dopamine receptors. *J. Neurochem.* 68:1990–97
121. Schetz JA, Sibley DR. 2001. The binding-site crevice of the D4 dopamine receptor is coupled to three distinct sites of allosteric modulation. *J. Pharmacol. Exp. Ther.* 296:359–63

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

122. Swaminath G, Lee TW, Kobilka B. 2003. Identification of an allosteric binding site for Zn^{2+} on the beta2 adrenergic receptor. *J. Biol. Chem.* 278:352–56

123. Fowler CB, Pogozheva ID, LeVine H, Mosberg HI. 2004. Refinement of a homology model of the mu-opioid receptor using distance constraints from intrinsic and engineered zinc-binding sites. *Biochemistry* 43:8700–10

124. Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D. 1992. Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.* 41:549–60

125. Galvez T, Urwyler S, Prezeau L, Mosbacher J, Joly C, et al. 2000. Ca(2+) requirement for high-affinity gamma-aminobutyric acid (GABA) binding at GABA(B) receptors: involvement of serine 269 of the GABA(B)R1 subunit. *Mol. Pharmacol.* 57:419–26

126. Conigrave AD, Quinn SJ, Brown EM. 2000. L-amino acid sensing by the extracellular Ca^{2+}-sensing receptor. *Proc. Natl. Acad. Sci. USA* 97:4814–19

127. Massot O, Rousselle JC, Fillion MP, Grimaldi B, Cloez-Tayarani I, et al. 1996. 5-hydroxytryptamine-moduline, a new endogenous cerebral peptide, controls the serotonergic activity via its specific interaction with 5-hydroxytryptamine1B/1D receptors. *Mol. Pharmacol.* 50:752–62

128. Massot O, Rousselle JC, Grimaldi B, Cloez-Tayarani I, Fillion MP, et al. 1998. Molecular, cellular and physiological characteristics of 5-HT-moduline, a novel endogenous modulator of 5-HT1B receptor subtype. *Ann. N.Y. Acad. Sci.* 861:174–82

129. Mishra RK, Makman MH, Costain WJ, Nair VD, Johnson RL. 1999. Modulation of agonist stimulated adenylyl cyclase and GTPase activity by L-proL-leu-glycinamide and its peptidomimetic analogue in rat striatal membranes. *Neurosci. Lett.* 269:21–24

130. Verma V, Mann A, Costain W, Pontoriero G, Castellano JM, et al. 2005. Modulation of agonist binding to human dopamine receptor subtypes by L-prolyl-L-leucyl-glycinamide and a peptidomimetic analog. *J. Pharmacol. Exp. Ther.* 315:1228–36

131. Fryer AD, Jacoby DB. 1992. Function of pulmonary M2 muscarinic receptors in antigen-challenged guinea pigs is restored by heparin and poly-L-glutamate. *J. Clin. Invest.* 90:2292–98

132. Hedlund PB, Carson MJ, Sutcliffe JG, Thomas EA. 1999. Allosteric regulation by oleamide of the binding properties of 5-hydroxytryptamine7 receptors. *Biochem. Pharmacol.* 58:1807–13

133. Thomas EA, Carson MJ, Neal MJ, Sutcliffe JG. 1997. Unique allosteric regulation of 5-hydroxytryptamine receptor-mediated signal transduction by oleamide. *Proc. Natl. Acad. Sci. USA* 94:14115–19

134. Grazzini E, Guillon G, Mouillac B, Zingg HH. 1998. Inhibition of oxytocin receptor function by direct binding of progesterone. *Nature* 392:509–12

135. Gimpl G, Burger K, Fahrenholz F. 1997. Cholesterol as modulator of receptor function. *Biochemistry* 36:10959–74

136. Politowska E, Kazmierkiewicz R, Wiegand V, Fahrenholz F, Ciarkowski J. 2001. Molecular modelling study of the role of cholesterol in the stimulation of the oxytocin receptor. *Acta Biochim. Pol.* 48:83–93
137. Borda E, Pascual J, Cossio P, De La Vega M, Arana R, Sterin-Borda L. 1984. A circulating IgG in Chagas' disease which binds to beta-adrenoceptors of myocardium and modulates their activity. *Clin. Exp. Immunol.* 57:679–86
138. Goin JC, Borda E, Segovia A, Sterin-Borda L. 1991. Distribution of antibodies against beta-adrenoceptors in the course of human *Trypanosoma cruzi* infection. *Proc. Soc. Exp. Biol. Med.* 197:186–92
139. Goin JC, Leiros CP, Borda E, Sterin-Borda L. 1997. Interaction of human chagasic IgG with the second extracellular loop of the human heart muscarinic acetylcholine receptor: functional and pathological implications. *FASEB J.* 11:77–83
140. Borda E, Camusso JJ, Perez Leiros C, Bacman S, Hubscher O, et al. 1996. Circulating antibodies against neonatal cardiac muscarinic acetylcholine receptor in patients with Sjogren's syndrome. *Mol. Cell Biochem.* 163–164:335–41
141. Bacman SR, Berra A, Sterin-Borda L, Borda ES. 1998. Human primary Sjogren's syndrome autoantibodies as mediators of nitric oxide release coupled to lacrimal gland muscarinic acetylcholine receptors. *Curr. Eye Res.* 17:1135–42
142. Ganzinelli S, Borda T, Sterin-Borda L. 2006. Regulation of m1 muscarinic receptors and nNOS mRNA levels by autoantibodies from schizophrenic patients. *Neuropharmacology* 50:362–71
143. Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, et al. 1999. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. *J. Clin. Invest.* 103:945–52
144. Elies R, Fu LX, Eftekhari P, Wallukat G, Schulze W, et al. 1998. Immunochemical and functional characterization of an agonist-like monoclonal antibody against the M2 acetylcholine receptor. *Eur. J. Biochem.* 251:659–66
145. Mobini R, Magnusson Y, Wallukat G, Viguier M, Hjalmarson A, Hoebeke J. 1999. Probing the immunological properties of the extracellular domains of the human beta(1)-adrenoceptor. *J. Autoimmun.* 13:179–86
146. Peter JC, Eftekhari P, Billiald P, Wallukat G, Hoebeke J. 2003. scFv single chain antibody variable fragment as inverse agonist of the beta2-adrenergic receptor. *J. Biol. Chem.* 278:36740–47
147. Peter JC, Wallukat G, Tugler J, Maurice D, Roegel JC, et al. 2004. Modulation of the M2 muscarinic acetylcholine receptor activity with monoclonal anti-M2 receptor antibody fragments. *J. Biol. Chem.* 279:55697–706
148. Bozon V, Di Scala E, Eftekhari P, Hoebeke J, Lezoualc'h F, et al. 2002. Agonist-like activity of antibodies directed against the second extracellular loop of the human cardiac serotonin 5-HT4(e) receptor in transfected COS-7 cells. *Receptors Channels* 8:113–21
149. Verdot L, Bertin B, Guilloteau D, Strosberg AD, Hoebeke J. 1995. Characterization of pharmacologically active antipeptide antibodies directed against the first and second extracellular loops of the serotonin 5-HT1A receptor. *J. Neurochem.* 65:319–28

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

Annual Review of
Pharmacology and
Toxicology

Volume 47, 2007

Contents

Allosteric Modulation of G Protein–Coupled Receptors
Lauren T. May, Katie Leach, Patrick M. Sexton, and Arthur Christopoulos 1

Pharmacogenomic and Structural Analysis of Constitutive G Protein–Coupled Receptor Activity
Martine J. Smit, Henry F. Vischer, Remko A. Bakker, Aldo Jongejan, Henk Timmerman, Leonardo Pardo, and Rob Leurs 53

Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway
Thomas W. Kensler, Nobunao Wakabayashi, and Shyam Biswal 89

Cell Signaling and Neuronal Death
Makoto R. Hara and Solomon H. Snyder 117

Mitochondrial Oxidative Stress: Implications for Cell Death
Sten Orrenius, Vladimir Gogvadze, and Boris Zhivotovsky 143

AMP-Activated Protein Kinase as a Drug Target
D. Grahame Hardie 185

Intracellular Targets of Matrix Metalloproteinase-2 in Cardiac Disease: Rationale and Therapeutic Approaches
Richard Schulz 211

Arsenic: Signal Transduction, Transcription Factor, and Biotransformation Involved in Cellular Response and Toxicity
Yoshito Kumagai and Daigo Sumi 243

Aldo-Keto Reductases and Bioactivation/Detoxication
Yi Jin and Trevor M. Penning 263

Carbonyl Reductases: The Complex Relationships of Mammalian Carbonyl- and Quinone-Reducing Enzymes and Their Role in Physiology
Udo Oppermann 293

Drug Targeting to the Brain
A.G. de Boer and P.J. Gaillard 323

Mechanism-Based Pharmacokinetic-Pharmacodynamic Modeling: Biophase Distribution, Receptor Theory, and Dynamical Systems Analysis
Meindert Danhof, Joost de Jongh, Elizabeth C.M. De Lange, Oscar Della Pasqua, Bart A. Ploeger, and Rob A. Voskuyl 357

Annu. Rev. Pharmacol. Toxicol. 2007.47:1-51.

The Functional Impact of SLC6 Transporter Genetic Variation
Maureen K. Hahn and Randy D. Blakely 401

mTOR Pathway as a Target in Tissue Hypertrophy
Chung-Han Lee, Ken Inoki, and Kun-Liang Guan 443

Diseases Caused by Defects in the Visual Cycle: Retinoids as Potential Therapeutic Agents
Gabriel H. Travis, Marcin Golczak, Alexander R. Moise, and Krzysztof Palczewski ... 469

Idiosyncratic Drug Reactions: Current Understanding
Jack Uetrecht 513

Non-Nicotinic Therapies for Smoking Cessation
Eric C.K. Siu and Rachel F. Tyndale 541

The Obesity Epidemic: Current and Future Pharmacological Treatments
Karl G. Hofbauer, Janet R. Nicholson, and Olivier Boss 565

Circadian Rhythms: Mechanisms and Therapeutic Implications
Francis Levi and Ueli Schibler 593

Targeting Antioxidants to Mitochondria by Conjugation to Lipophilic Cations
Michael P. Murphy and Robin A.J. Smith 629

Acute Effects of Estrogen on Neuronal Physiology
Catherine S. Woolley 657

New Insights into the Mechanism of Action of Amphetamines
Annette E. Fleckenstein, Trent J. Volz, Evan L. Riddle, James W. Gibb, and Glen R. Hanson 681

Nicotinic Acetylcholine Receptors and Nicotinic Cholinergic Mechanisms of the Central Nervous System
John A. Dani and Daniel Bertrand 699

Contrasting Actions of Endothelin ET_A and ET_B Receptors in Cardiovascular Disease
Markus P. Schneider, Erika I. Boesen, and David M. Pollock 731

Indexes

Cumulative Index of Contributing Authors, Volumes 43–47 761

Cumulative Index of Chapter Titles, Volumes 43–47 764

Errata

An online log of corrections to *Annual Review of Pharmacology and Toxicology* chapters (if any, 1997 to the present) may be found at http://pharmtox.annualreviews.org/errata.shtml