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A kinetic view of GPCR allostery and biased agonism

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G-protein-coupled receptors (GPCRs) are one of the most tractable classes of drug targets. These dynamic proteins can adopt multiple active states that are linked to distinct functional outcomes. Such states can be differentially stabilized by ligands interacting with the endogenous agonist-binding orthosteric site and/or by ligands acting via spatially distinct allosteric sites, leading to the phenomena of 'biased agonism' or 'biased modulation'. These paradigms are having a major impact on modern drug discovery, but it is becoming increasingly apparent that 'kinetic context', at the level of both ligand–receptor and receptor—signal pathway kinetics, can have a profound impact on the observation and quantification of these phenomena. The concept of kinetic context thus represents an important new consideration that should be routinely incorporated into contemporary chemical biology and drug discovery studies of GPCR bias and allostery.

GPCRs are the largest family of cell-surface signal transducers[1]. The GPCR family recognizes a diverse array of endogenous agonists despite possessing a common canonical fold of seven transmembrane (TM) helical segments connected by three extracellular loops (ECLs) and three intracellular loops (ICLs). Conformational changes that occur upon agonist activation allow coupling of the GPCR to heterotrimeric G proteins to elicit production of second messengers, activation of kinase cascades and modulation of ion channels. Regulatory processes, such as recruitment of β-arrestins, receptor phosphorylation, desensitization and internalization, can influence the duration and magnitude of signal transduction. In addition, β-arrestins can act as scaffolding and transducer proteins, recruiting intracellular effectors to elicit alternative waves of signaling[2].

Although GPCR drug discovery has predominantly focused on targeting the endogenous agonist-binding orthosteric site, GPCRs also have additional spatially distinct, yet conformationally linked, allosteric sites[3]. Ligands that bind to allosteric sites offer a number of advantages over orthosteric drugs. These benefits include a higher likelihood of receptor subtype selectivity, the potential to 'fine tune' endogenous agonism while maintaining physiological spatiotemporal signaling patterns, and having a 'ceiling level' to the allosteric effect determined by the magnitude and direction of the cooperativity between orthosteric and allosteric sites, resulting in a lower potential for on-target overdose[3]. Allosteric ligands that enhance orthosteric agonist activity are termed positive allosteric modulators (PAMs), those that inhibit orthosteric agonism are called negative allosteric modulators (NAMs), and those that occupy an allosteric site and have no net effect on the orthosteric ligand at equilibrium are referred to as neutral allosteric ligands (NALs)[3]. Allosteric ligands can also act as agonists or inverse agonists in their own right[4]. Given such a rich tapestry of chemical probes and potential leads for targeting of GPCRs, it is not surprising that these proteins remain preeminent contemporary drug targets[1].

The simplest mechanism of GPCR activation is a two-state conformational selection model, whereby the receptor exists in an equilibrium between active and inactive states; agonists shift the equilibrium toward the active state[5], whereas inverse agonists shift the equilibrium to the inactive state[6]. The higher the relative affinity for the active state over the inactive state, the higher the 'efficacy' of a given agonist. Within such a scheme, absolute differences in ligand efficacies can be accounted for by differences in receptor expression and/or stimulus-response coupling between different cells or organs, but the rank orders of compound efficacies and potencies are retained[7]. This is an important distinction, because in drug discovery programs initial evaluations of efficacy typically involve the measurement of a single pathway downstream of receptor activation, usually in a high-throughput manner. Within a two-state model, comparison of relative drug activities in one type of system should be predictive of relative activities in another, including the therapeutic endpoint. The model can also provide the simplest explanation for 'probe dependence', a unique property associated with allosteric modulators whereby the magnitude and direction of the allosteric effect can change depending on the orthosteric ligand that is used to probe receptor function. Specifically, the magnitude of the allosteric effect will change with the efficacy of the orthosteric ligand, with higher efficacy agonists being potentiated to a greater extent than low efficacy agonists, whereas inverse agonists are actually inhibited by the modulator[8]. Therefore, within a two-state scheme, PAMs of agonists would manifest as NAMs of inverse agonists, and vice versa[8].

It is now apparent, however, that different ligands acting at the same receptor in the same cellular background can stabilize distinct receptor conformations such that only a subset of the possible signaling repertoires mediated by the receptor are involved to the relative exclusion of others (**Fig. 1**)[9,10]. This phenomenon is termed 'biased agonism', 'functional selectivity' or 'ligand-directed signaling', and is indicative of the fact that GPCRs can adopt more than one active state. Similarly, there is an increasing array of allosteric ligands with different degrees of modulation that vary dramatically in a probe- and pathway-specific manner that cannot be accounted for by simple differences in orthosteric ligand efficacy or stimulus-response coupling—a phenomenon termed 'biased modulation' (**Fig. 1**)[11,12]. The promise of biased agonism and modulation is discovery of drugs that selectively engage therapeutically relevant signaling pathways mediated by a receptor while avoiding those that contribute to undesirable on-target side effects[13,14]. Given that such observations imply the existence of multiple functionally relevant

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GPCR states, *in vitro* measurement of relative efficacies, potencies or degrees of allosteric modulation that are based upon a single cellular readout may not be predictive of activity in a different cellular, tissue or *in vivo* context. This can lead to translational failures, because the link between preclinical activity determined through the study of a given signaling pathway and the actual pathophysiology being targeted remains poorly defined for the majority of diseases. Thus, contemporary GPCR chemical biology and drug-discovery programs must now acknowledge the influence of biased agonism or biased modulation[9,10,12,13]. This Perspective focuses on the influence of kinetic context, as defined by both ligand-binding kinetics and receptor-signaling kinetics, on biased agonism and on allosteric modulation of GPCRs.

Quantifying bias and allostery

Although biochemical, biophysical and structural studies are beginning to provide relatively direct evidence of ligand-dependent conformational states of GPCRs[15–23] and their associated transducers[24–26], the majority of research supporting conformation-driven mechanisms of bias for novel ligands relies on indirect, yet more facile, cell-signaling and pharmacological data. This, in turn, has necessitated the development of analytical methodologies that can be applied to experimental data to allow identification and quantification of biased agonism and allostery[27]. In general, the most widespread methods for this purpose are based on either 'operational models'[11,28] or thermodynamic linkage models, such as the classic 'ternary complex model' (TCM) (**Fig. 2**; **Box 1**)[29–31]. Irrespective of the method, application of such analytical approaches assumes a condition of equilibrium among all reactants. In reality, biological systems are dynamic. For example, the ternary complex of agonist, receptor and G protein is not stable and does not accumulate appreciably in the presence of agonist and GTP in whole cells. Furthermore, GPCRs themselves undergo cycles of endocytosis and recycling, among others. Thus, it is not clear to what extent equilibrium measurements might predict the full spatiotemporal repertoire of agonist action. The situation is especially pertinent to cell-based signaling assays, which, by their very nature, are unlikely to reflect an equilibrium state. As a consequence, the interplay between ligand and signaling kinetics, including studies of ligand 'residence time' (**Box 2**), are increasingly becoming a new focus in the detection and quantification of biased agonism and allosteric modulation.

Ligand-binding kinetics and agonist efficacy

GPCRs are allosteric proteins, as the conformational transition mediating signal transduction involves a reciprocal, cooperative interaction between the orthosteric ligand-binding site and an intracellular transducer that are typically around 40 Å apart. A classic hallmark of many allosteric interactions is the ability of one substance to alter the affinity of another through such cooperative effects, by changes in either dissociation rate or association rate, or both. Accordingly, early biochemical studies demonstrated that the dissociation rate of a radiolabeled agonist could be modified when the GPCR is uncoupled from the nucleotide-free G protein, suggesting that the interplay between the kinetics of agonist and transducer binding must play an integral role in efficacy. Indeed, the notion that agonist kinetics and efficacy might be linked is a concept that was first proposed nearly 60 years ago, when 'receptors' were still regarded as a theoretical construct rather than a physical entity. Specifically, a 'rate theory' of drug action was proposed based upon the observation that the efficacy and rate of actions for a series of ligands mediating contraction of the guinea pig ileum were correlated[32]. In this model, a high-efficacy ligand dissociates rapidly from the receptor, allowing another agonist to bind, whereas a slowly dissociating agonist effectively acts as a competitive antagonist, preventing agonist binding and therefore displaying lower efficacy. More contemporary models, incorporating the known biochemical properties

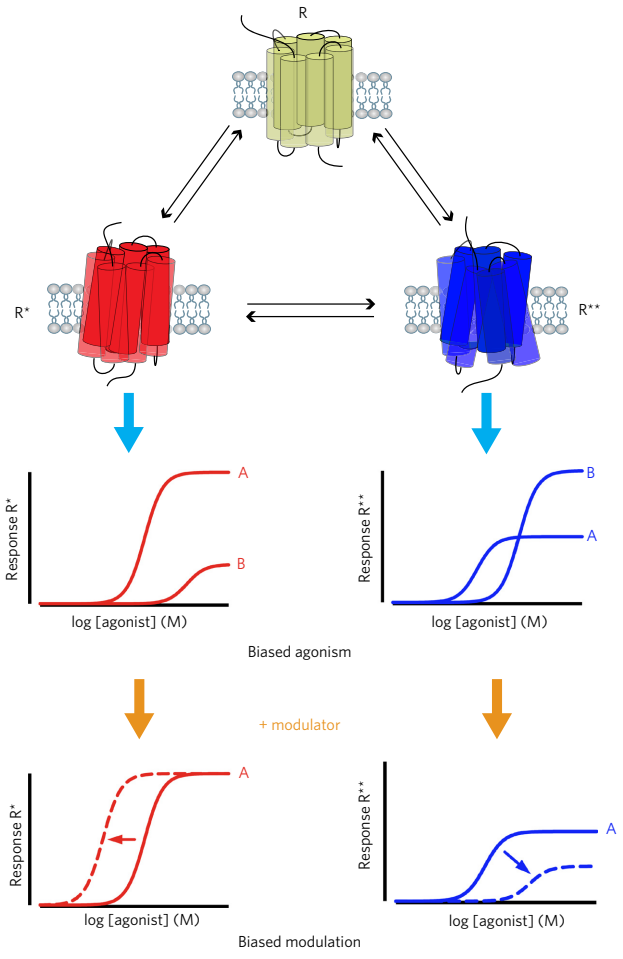

Figure 1 | Biased agonism and biased modulation. GPCRs can exist in a dynamic equilibrium between multiple inactive and active conformations (depicted here as a minimal scheme: R, inactive; R* or R**, active). Distinct active states can engage different transducer–effector systems and can be enriched in a ligand-dependent manner at the relative expense of other states, either by the binding of a single ligand (biased agonism) or the co-binding of an allosteric modulator (biased modulation). A and B denote two different orthosteric agonists, whereas the dashed curves indicate examples of pathway-biased modulation on agonist A.

GPCRs, have challenged this view, but continue to highlight the role of kinetics as determinants of GPCR efficacy. For example, a model was proposed in which the receptor essentially acts as an 'enzyme' that catalyzes nucleotide exchange on the G protein and the agonist acts as a 'PAM', facilitating this process[33]. Extensions to this model have included recycling of the G protein[34,35]. In contrast to the original rate theory, agonist residence time in these models is predicted to be positively correlated with efficacy, because the longer an agonist remains bound to the receptor the more cycles of G-protein activation it may catalyze.

Since the turn of the millennium, structural, biophysical and biochemical studies have provided unprecedented molecular-level insights into the allosteric transitions governing GPCR signal transduction and the role of dynamics in these processes[36–41]. In general, these studies have revealed that the largest conformational changes between the inactive and the active states occur within the intracellular portion of the GPCR, including a large outward movement of TM domain 6 (varying from 11 to 16 Å depending on the GPCR) and a conformational rearrangement of TM domains 5 and 7 close to the NPxxY motif, to facilitate transducer binding. Of particular note was a recent study using purified β_2-adrenergic receptor

in complex with heterotrimeric G_s protein and a nanobody, Nb80, which recognizes the G-protein site and stabilizes the active conformation, demonstrating that the nucleotide-free G protein and Nb80 substantially decreased the rate of orthosteric ligand association and dissociation. This provides direct evidence that G-protein binding engenders a 'closed' conformation of the orthosteric pocket[42]. By contrast, inverse agonist and G-protein binding to the β_2-adrenergic receptor appeared mutually exclusive, in accordance with the action of inverse agonists to decrease GPCR constitutive activity.

Collectively, the aforementioned studies have stimulated new research exploring potential correlations between agonist efficacy and residence time at GPCRs. For example, a tight correlation was found between the dissociation rate constants of a range of agonists at the M_3 muscarinic acetylcholine receptor (mAChR), and their efficacy was determined in both [^{35}S]GTPγS and calcium mobilization assays[43]. A similar correlation was observed for agonists at the adenosine A2a receptor ($A_{2A}AR$)[44]. The slow dissociation of the β_2 adrenergic agonist C26 was proposed to contribute to its superior efficacy relative to epinephrine[45]. However, similar studies at the adenosine A_1 and dopamine D_2 receptors found no such relationships[46,47]. Thus, further studies are needed to clarify the relationship between efficacy and residence time, because the lifetime of the receptor–agonist complex may differentially influence coupling to one signaling pathway over another for certain GPCRs but perhaps not for others.

The kinetics of GPCR allosteric modulator mechanisms

Although it is well accepted that GPCRs possess spatially distinct allosteric sites recognized by both synthetic and endogenous substances[48,49], it is often overlooked that the designation of an allosteric modulator as a 'PAM', a 'NAM' or a 'NAL' is not absolute but, rather, conditional depending on at least two key considerations. The first is with regards to the orthosteric probe and the pathway being interrogated in the presence of the allosteric ligand[3]. The second is that the classifications themselves reflect the net effect of the modulator at equilibrium. Allosteric modulator effects can be manifested at the level of orthosteric ligand affinity and/or orthosteric ligand efficacy; the former represents the simplest mechanism of allosteric modulation and is adequately quantified by the 'allosteric ternary complex model' (ATCM; **Box 1**). Because affinity, an equilibrium parameter, is defined by the ratio of ligand association and dissociation rate constants, modulation of orthosteric ligand affinity in either a positive (PAM) or negative (NAM) direction can arise

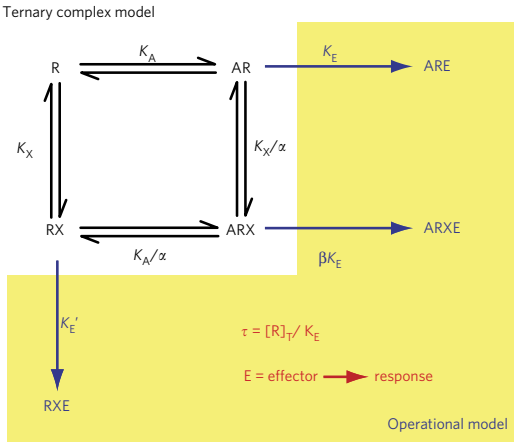

Figure 2 | Common analytical models of ligand action at GPCRs. The 'ternary complex model' is the simplest mass-action scheme that can be applied to describe the interaction of a GPCR (R) with its orthosteric ligand (A) and either a transducer protein or an allosteric modulator (X), with the binding of each species being governed by their respective concentrations and equilibrium dissociation constants (K_A and K_X). The affinity of A or X is reciprocally modified in the same direction and to the same extent by the co-binding of the other, which is quantified by the cooperativity factor, α. This mass-action binding scheme can be extended into an 'operational model' of agonism and/or allostery by linking the initial receptor-bound complexes to a final, experimentally determined, effector-response pathway by treating the entire signaling cascade as a 'virtual' Michaelis–Menten scheme. This linkage is governed by K_E, the 'virtual' equilibrium dissociation constant of the agonist–receptor complex for coupling to the observed cellular response, and receptor density, $[R]_T$. Thus, the ratio of $[R]_T / K_E$ can be used as an overall operational measure of agonist efficacy, τ. In the presence of an allosteric modulator, agonist signaling efficacy can potentially be modified by the scaling factor, β, in addition to any potential effects on binding affinity governed by α.

Box 1 | Quantification of bias and allostery

For quantification of biased agonism using operational models, key parameters used are the equilibrium dissociation constant of an agonist for its receptor (K_A) and an operational measure of its signaling efficacy, τ, which embodies both the 'intrinsic' efficacy of the molecule and the sensitivity of the overall system to agonism[86]. The ratio of these two parameters (τ/K_A), termed the 'transduction coefficient', is used as an index of the overall activity of an agonist at a given pathway and represents a starting point for bias quantification. Normalization of this value to that of a reference agonist obtained at the same pathway is vital, as it theoretically minimizes or removes the unwanted influence of additional system factors (for example, cellular background, effector–transducer stoichiometry and coupling efficiency) and assay conditions. Other methods, such as the 'relative activity scale' (E_{max}/EC_{50}) or the 'sigma' method are based on similar theoretical underpinnings[87,88]. The extension of operational models to accommodate the action of allosteric modulators require the incorporation of additional parameters, such as the dissociation constant (K_B) and operational efficacy (τ_B) of the allosteric ligand as well as, importantly, the overall allosteric effect of the modulator on orthosteric agonist affinity (α) or efficacy (β)[11]. In brief, $\alpha\beta > 1$ defines a PAM effect, values of $0 < \alpha\beta < 1$ define a NAM effect, whereas $\alpha\beta = 1$ defines a NAL. In these models, biased modulation is manifested by $\alpha\beta$ values that are signal-pathway dependent in a manner not linked to the strength of coupling of the GPCR to each pathway[8,11].

In contrast to operational approaches, thermodynamic linkage models propose explicit mass action mechanisms to define and quantify bias and allostery. For example, a recent study generated different angiotensin II type 1 receptor fusion proteins with either a G protein or β-arrestin in heterologous cells and used radioligand competition assays to quantify a high (K_{Hi}) affinity state, associated with the receptor–transducer fusion, and a low-affinity state (K_{Lo}), associated with the uncoupled receptor for agonists of varying efficacies. Based on the thermodynamic reciprocity embodied in the TCM, the K_{Hi}/K_{Lo} ratio for each agonist was defined as a measure of 'molecular efficacy', which correlated well with separate measures of G-protein- and β-arrestin-mediated efficacy[89]. The same TCM, sometimes referred to as the 'allosteric' TCM (ATCM) to differentiate it from the TCM of agonist–receptor–G protein, has been applied to the interaction between orthosteric and allosteric ligands to quantify the magnitude of allosteric modulation of ligand affinity (α). However, extensions of this model to quantify separate allosteric effects on ligand signaling efficacy are not common; operational approaches remain the mainstay in this instance[11,30,90].

Box 2 | Residence time as a predictor of drug action

The *in vitro* characterization of compounds as part of drug discovery programs has often focused on equilibrium measurements of drug–target binding affinity. However, the concentration of a drug available for interaction with its target will be in constant flux driven by processes such as gastrointestinal absorption, tissue distribution, renal metabolism and hepatic clearance. Equilibrium measurements of affinity may not predict the *in vivo* effect of a drug under such nonequilibrium conditions[91]. Instead, the duration of the drug–target complex, or residence time, may be the key determinant of biological activity. Indeed, residence time has been used as a specific focus in the development of a number of drugs entering clinical development[91]. If one considers a 1:1 binding interaction between one receptor molecule and one molecule of drug then the affinity (K_d) is defined by:

$$K_d = \frac{k_{off}}{k_{on}}$$

The dissociation rate constant (k_{off}) is dependent on receptor–ligand interactions in the binding pocket. By contrast, the observed rate of association of a ligand depends not only upon the intrinsic association rate constant (k_{on}), but also upon the concentration of ligand, and will be limited by the rate of diffusion between the two binding partners (typically 10^8 to 10^9 $M^{-1}s^{-1}$). Residence time (defined as $1/k_{off}$) is driven by the rate of dissociation[91]. However, residence time and target affinity need not necessarily be correlated due to a variance of the k_{on} among a set of compounds. Indeed, structural changes to a ligand may have compensatory effects upon both k_{on} and k_{off} such that molecules with a common pharmacophore series can exhibit similar affinities but vastly different residence time[92,93]. k_{on} may play an additional role in prolonging the apparent lifetime of a drug–receptor complex through the process of rebinding in conditions of limited diffusion[94]. The relationship between *in vivo* efficacy and ligand residence time has begun to be explored at a number of GPCR targets[95]. For example, the long-lasting action of the antagonist tiotropium for the treatment of chronic obstructive pulmonary disease has been proposed to be derived from its slow dissociation rate from the muscarinic M_3 acetylcholine receptor[96]. However, a prolonged receptor residence time may not always be desirable, for example the slow dissociation rate of typical antipsychotics from the dopamine D_2 receptor has been linked to extrapyramidal side effects and prolactin release[97].

via a change in orthosteric ligand association or dissociation, or both[50]. However, it is also important to note that ligands competing for the same (for example, orthosteric) site as well as those targeting spatially distinct allosteric sites can both influence the 'observed' association of an orthosteric ligand as the system approaches equilibrium[51], thus complicating the use of association kinetics to ascribe a mechanism of action to putative small-molecule modulators. In contrast, the only mechanism by which the dissociation rate of a preformed ligand–receptor complex can be modified is a conformational change mediated by the binding of a second ligand to a different site. This is why dissociation kinetic experiments remain a hallmark feature of studies of allostery (**Fig. 3**).

Kinetic studies of GPCR allostery have identified mechanisms of action, as well as numerous caveats associated with the interpretation of kinetic data. For example, dissociation kinetic studies are a 'one-way' experiment; the demonstration that one ligand can alter the dissociation rate of another ligand can be taken as presumptive evidence of an allosteric mechanism, but a lack of effect on probe dissociation cannot be taken as evidence of an absence of an allosteric mechanism for at least three reasons. First, if the modulator is a strong NAM of orthosteric ligand affinity, and because allosteric interactions are thermodynamically reciprocal, it may simply be experimentally impossible to achieve sufficient occupancy of the allosteric site by the NAM in the presence of the pre-equilibrated orthosteric probe to observe any effects on kinetics due to high negative cooperativity[52]. Second, if the allosteric modulator exerts its effects predominantly on orthosteric ligand efficacy rather than affinity, it would be difficult to detect in a dissociation kinetic assay, which is biased toward affinity modulators. Finally, a dissociation kinetic assay will not detect allosteric modulators that mediate effects solely through changes in orthosteric ligand association[53].

Nonetheless, kinetic assays to study allosteric modulators remain extremely useful if the aforementioned caveats are acknowledged and they are combined with appropriate analytical approaches, both for detection and validation of their mechanism (**Fig. 3**). Prototypical examples include the use of dissociation kinetic assays to validate allosteric mechanisms of 5-(*N*,*N*-hexamethylene)amiloride, a NAM of the antagonist [^{3}H]ZM241285 at the A_{2A}AR[54] (**Fig. 3a**), or PD 117,975, a PAM of agonists at the adenosine A_1 receptor (A_1AR)[55] (**Fig. 3b**); in each instance, the observed direction of effect of the allosteric modulator on orthosteric ligand dissociation is sufficient to explain the equilibrium properties of the modulator. However, more complex examples exist. For instance, gallamine is a NAM of both agonist and antagonist equilibrium binding at M_2 mAChRs that, nonetheless, retards orthosteric ligand dissociation[31,56]. This effect appears to be counterintuitive, unless it is appreciated that the modulator also slows down the association rate of orthosteric ligands at lower concentration ranges (i.e., with higher potency) relative to the concentrations required to slow dissociation; i.e., gallamine (and related compounds) is a NAM because it has a higher affinity for the allosteric site on the free receptor relative to its affinity for the receptor occupied by orthosteric ligand[30,50]. The opposite is the case for PAMs. These are important considerations, because a focus on the direction of an allosteric effect on orthosteric ligand dissociation rate (acceleration or retardation) alone cannot necessarily be used as a predictor of its effect at equilibrium unless the effects on orthosteric association are also known. Indeed, an extreme example can be seen with NALs. For instance, the allosteric ligand LY2033298 has a minimal effect on the equilibrium binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) at the M_4 mAChR, which could be interpreted as either a lack of activity or a result of NAL behavior. The demonstration that LY2033298 can, over a similar concentration range, completely retard both the dissociation and the association rates of [^{3}H]NMS, however, was used to conclude the presence of an allosteric mode of interaction as well as provide the mechanistic basis for its NAL effect, i.e., no net change in [^{3}H]NMS affinity at equilibrium (**Fig. 3c**)[57].

As seen in studies focusing on the interplay between agonist—transducer kinetics and signaling efficacy, structural and computational breakthroughs are yielding high-resolution insights into small-molecule GPCR allostery, with arguably the best example being the mAChR family. The crystallographic solution of inactive states of four (M_1–M_4) mAChR subtypes identified a 'common' extracellular vestibule above the orthosteric binding pocket that contributes to the allosteric site[40,41,58,59]. This region is characterized by divergent amino acids, relative to the completely conserved orthosteric pocket, and varying electrostatic surface potentials[58], which may account for some of the differences in subtype selectivity that are observed with allosteric modulators of this GPCR family. Comparison of active-state structures of an agonist-bound M_2

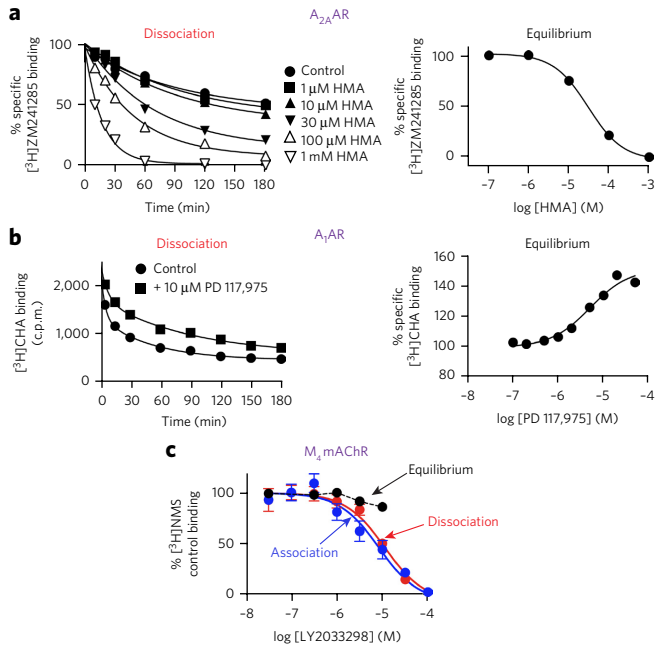

Figure 3 | Effects of allosteric-modulator-mediated changes in binding kinetics on the equilibrium behavior of orthosteric ligands. (**a**) 5-(*N*,*N*-hexamethylene)amiloride (HMA) is a negative allosteric modulator (NAM) of the equilibrium binding of the antagonist [^{3}H]ZM241285 at the A_{2A}AR, because it increases the dissociation rate of the radioligand. Image adapted with permission from ref. 54. (**b**) PD 117,975 is a positive allosteric modulator (PAM) of the equilibrium binding of the agonist [^{3}H]CHA at the A_1AR through mediating a decrease in agonist dissociation rate. Image adapted with permission from ref. 55. (**c**) LY2033298 is a neutral allosteric ligand (NAL) of the equilibrium binding of the antagonist [^{3}H]NMS at the M_4 mAChR, by decreasing both association and dissociation rates of the orthosteric ligand to the same extent over the same concentration range. Image adapted from ref. 57.

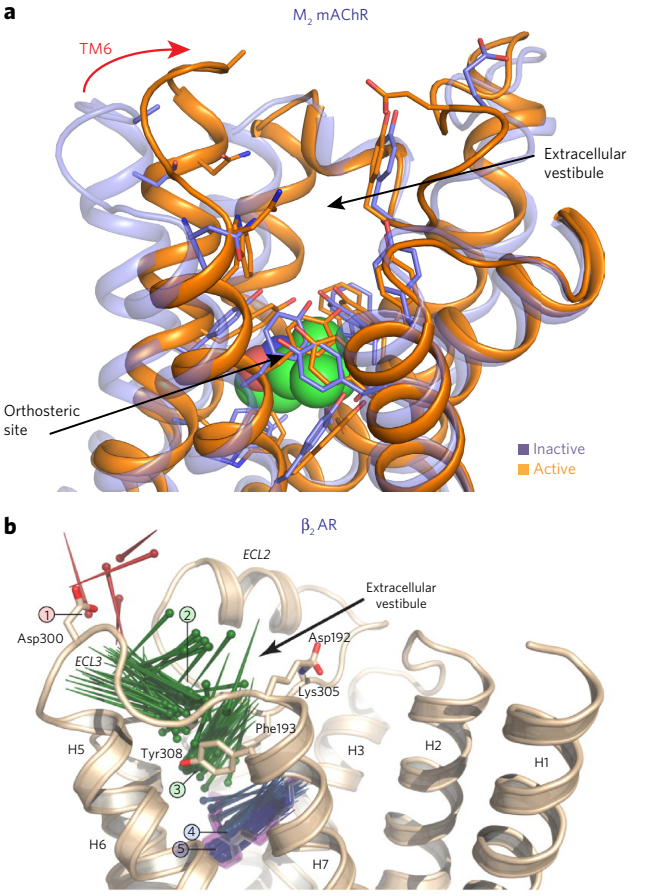

Figure 4 | The extracellular vestibule of rhodopsin-like GPCRs can control the access and egress of orthosteric ligands, and can contribute to the formation of a binding site for allosteric modulators. (**a**) Comparison of the extracellular interface of the M_2 mAChR in an inactive state (PDB 3UON) and an active state (PDB 4MQS) bound to the agonist iperoxo (green). The extracellular vestibule, which also forms an allosteric binding site, contracts considerably during activation, an effect largely governed by a rigid body movement of TM6 (refs. 40,41). (**b**) Molecular dynamics simulations of the binding pathway of the orthosteric antagonist alprenolol at the β_2AR reveal a passage through several metastable states, moving from bulk solvent (red pins, pose 1) into the extracellular vestibule (green pins, poses 2 and 3), and finally into the orthosteric binding pocket (blue pins, poses 4 and 5). ECL, extracellular loop. Image reproduced with permission from ref. 61.

mAChR in the absence and presence of the PAM LY2119620 to an inactive, antagonist-bound state revealed striking differences in the intracellular regions, the orthosteric pocket and the extracellular vestibule housing the allosteric site, which contracts dramatically during the transition between inactive to active states[41] (**Fig. 4a**). However, a recent study of the M_4 mAChR suggested that there may be additional mechanisms contributing to the transmission of cooperativity that are not fully captured in the current crystal structures[58]. This possibility is in accord with findings from long time-scale molecular dynamics simulations of allosteric modulators at the inactive-state M_2 mAChR[60].

Structural insights into the mode and action of mAChR allosteric modulators also reveal a mechanistic basis for the effect of such modulators on orthosteric ligand-binding kinetics. As they occupy a pocket above the orthosteric binding site of the mAChRs, it is not surprising that these compounds change orthosteric ligand association and/or dissociation, and it is likely this mechanism extends to related GPCRs. For instance, molecular dynamics studies of β_1 and β_2 adrenergic receptors, M_3 mAChRs and A_1ARs suggest that even orthosteric ligands traverse a pathway that includes metastable binding to an extracellular vestibule, essentially acting as a 'way station' before final engagement with the deeper orthosteric site (**Fig. 4b**)[59,61,62]. One can envisage, therefore, that certain classes of molecules may prefer to remain within the vestibule, thus acting as allosteric modulators. These types of modulators can exert profound effects on the kinetics of access and egress of orthosteric ligands as the system approaches equilibrium, which was indeed directly observed at the mAChRs (**Fig. 5a**,**b**). In some cases, a kinetic 'trapping' mechanism of such allosteric ligands may be so profound that the system fails to attain equilibrium over the time course of a typical experiment, yielding complex orthosteric binding curves that can be mechanistically misinterpreted (**Fig. 5c**)[63,64]. In such instances, application of kinetic variants of the ATCM can account for and quantify these effects[64].

Although allosteric effects on orthosteric ligand kinetics are often considered within the context of a single GPCR, there is increasing evidence to suggest that GPCRs can form dimers or higher-order oligomers, and that cooperativity may occur across the interface of two or more interacting protomers. Early evidence for such cooperativity was obtained from dissociation kinetic studies using β-adrenergic receptors[65]. Dissociation kinetics have subsequently been used to identify cooperative interactions across dimers or higher-order oligomers for a number of GPCRs, including adenosine, dopamine, glycoprotein hormone, chemokine and glucagon-like peptide 1 receptors[66–69]. More recent approaches

such as total internal reflection fluorescence microscopy, which can visualize and track fluorescently labeled GPCRs in live cells, and new mass spectrometric platforms[70] have been used to further investigate the oligomerization process. The latter recently showed a crucial role for specific interfacial lipids in the oligomerization of a subset of GPCRs[70]. As interactions across homomeric or heteromeric interfaces can influence GPCR allostery, these new studies suggest a possible modulatory role of membrane lipids in signaling by specific GPCRs.

The impact of kinetics on GPCR biased agonism

In contrast to the many studies that explore the effect of kinetics on allosteric modulators of GPCRs, far fewer studies address the impact of kinetic context on biased agonism. This was explicitly investigated very recently in a study of the action of agonists at the dopamine D_2 receptor[47]. Kinetic binding parameters of selected agonists were determined, their action at distinct signaling pathways was compared at different time points and biased agonism was quantified using the operational model described in **Box 1**. A key finding of the study was that kinetic context, defined both by ligand-binding kinetics and the kinetics associated with different cellular signaling processes, profoundly influenced observations of biased agonism. Indeed, for three ligands within this study, the direction of bias between two different assay endpoints was even reversed over time. Furthermore, the study demonstrated that kinetic context was the main mechanism underlying discrepancies in the biased actions of the antipsychotic aripiprazole that were observed in previously published studies[71,72].

Such findings add a layer of complexity to the interpretation of bias. They illustrate that analytical methods based on equilibrium models of agonism cannot always exclude confounding factors conferred by kinetic context. In particular, one must consider the implications of assuming equilibrium when applying such models to data obtained from experiments performed under nonequilibrium conditions, because a key component in the determination of bias is the estimation of agonist potency, which would be underestimated for slowly dissociating agonists when their action is measured at short time points[47,73]. Indeed, a recent study demonstrated that the potency of slowly dissociating ergot agonists at the serotonin $5HT_{2B}$ receptor was underestimated when transient signaling events were measured[74]. Of interest, such ergot derivatives were described as biased agonists, because they displayed different potencies in a transient assay calcium mobilization assay compared to those obtained in a β-arrestin recruitment assay measured following overnight incubation[74]. This bias was reconciled with the stabilization of distinct receptor conformations as revealed by X-ray crystallography studies[75,76]. It is possible that some of the limitations of short time-point assays may be overcome by newer, real-time functional assays, including 'label free' impedance-based approaches; however, even these studies need to consider the impact of dynamic events, such as receptor endocytosis, occurring within the time course of the assay. Finally, numerous studies have shown how the structure–activity relationships of GPCR ligands can be supplemented with measurements of biased agonism[77–80]. A general feature of these studies is that relatively subtle changes to the ligand can engender substantial changes in bias. It remains to be seen to what extent such changes in bias are caused by changes in ligand-binding kinetics.

It is important to note, however, that the above observations do not exclude biased agonism conferred by conformational selection. Indeed, the duration of a ligand–receptor complex, as determined by ligand-binding kinetics, may determine the different effector and regulatory proteins engaged over time (which themselves may vary in abundance/location depending on cell type) and, hence, the conformational landscape explored by the GPCR (**Fig. 6**). In support of this hypothesis, studies have found that GPCRs elicit sustained waves of signaling following internalization[81–83], and recent work has suggested a link between residence time and persistent signaling from internalized $5HT_{2B}$ and calcitonin receptors[74,84]. Most recently, the ability of lysergic acid diethylamide (LSD) to exhibit bias toward β-arrestin recruitment at $5HT_{2A}$ and $5HT_{2B}$ receptors was shown to be dependent upon its residence time[85]. Thus, rather than a 'confounding factor', kinetic context represents an important component that could be incorporated into the classification or selection of different biased agonists. Indeed, although the comparison of bias between rapid signaling events (such as intracellular calcium mobilization) and those that are more sustained are inherently subject to the influence of kinetic context, such comparisons may provide information on the key nature of a drug's effect. As a corollary, ascribing an absolute directionality to the pathway bias of an agonist may be an insufficient descriptor of the action of a drug, as the bias determined at a short time point may be significantly different from that detected after prolonged agonist stimulation. Thus, observations of bias should at least be qualified by the experimental context under which they were derived.

Of course, the potential for biased effects is not restricted to drugs that target the orthosteric pocket of GPCRs and pathway-biased allosteric drug action has been observed at a number of GPCRs[12]. However, as for orthosteric ligands, such observations should now also be viewed in light of the impact of kinetic context. In this regard, due to the reciprocal nature of allosteric interactions, one must consider both the impact of the binding kinetics of the allosteric modulator and orthosteric ligand alone, as well as the modulatory effects they exert upon each other.

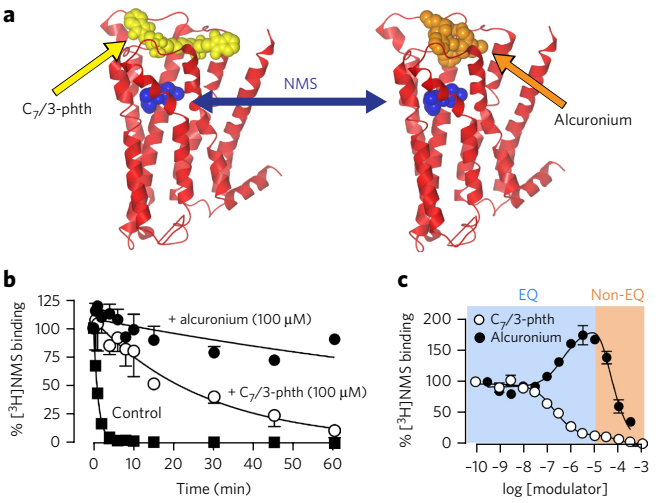

Figure 5 | Failure to attain equilibrium between orthosteric and allosteric ligands can lead to apparently complex behaviors of allosteric modulators. (**a**) Predicted poses of a NAM, C_7/3-phth, and a PAM, alcuronium, of the orthosteric antagonist NMS at the M_2 mAChR based on molecular dynamics simulations. In both instances, the allosteric modulators have the potential to 'cap' the orthosteric site, thus affecting access and egress of the orthosteric ligand. Image adapted from ref. 60. (**b**) Experimental validation that high concentrations of either C_7/3-phth or alcuronium can retard the dissociation of [^{3}H]NMS from the M_2 mAChR[64]. (**c**) Consequences of inadequate equilibration time between high concentrations of allosteric modulators and [^{3}H]NMS at the M_2 mAChR. The region in blue indicates effects observed at equilibrium (EQ), whereas that in orange reflects the fact that a nonequilibrium (non-EQ) state exists at the time of termination of the experiment, leading to the overall appearance of a biphasic binding isotherm for the NAM or a bell-shaped curve for the PAM. Images in **Figure 5b**,**c** adapted with permission from ref. 64.

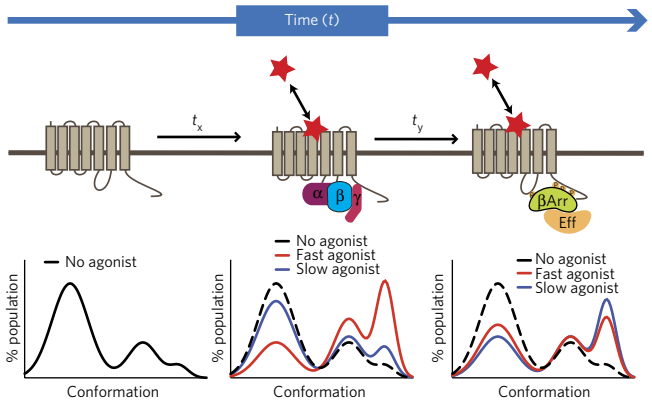

Figure 6 | Kinetic context can have a profound impact on the quantification of biased agonism. The impact of kinetic bias on the energy landscape of a GPCR. A receptor may adopt different conformations as it engages different signaling transducer and regulatory proteins. Agonists (red stars) may have different residence times (t_x or t_y) on the receptor. The duration of a ligand–receptor complex may determine the different proteins that can be engaged and thus the overall conformational landscape that can be explored by the agonist–receptor complex over time. βArr, β-arrestin; Eff, effector. Image reproduced from ref. 47.

How future studies of allostery and bias can incorporate kinetic context

It is clear that kinetic context can have a considerable impact on the interpretation of biased agonism. Similarly, ligand-binding kinetics are fundamental to the action of allosteric ligands at GPCRs. The consideration of kinetic context adds an additional challenge in efforts to correlate *in vitro* observations of biased agonism with *in vivo* measurements of physiological effects, a key step in any drug-discovery program. For example, it would be naive to expect that a bias profile detected in a model system using measurements of relatively acute agonist exposure would necessarily predict the *in vivo* action of a chronically added agonist. The ideal situation would be to use the physiologically relevant tissue to screen for biased ligands. However, such an approach is not practical for the screening of a large number of compounds. Furthermore, a detailed understanding of the relationship between different signaling pathways and the observed physiological effect is required to provide unequivocal evidence that it is the biased action of a ligand leading to a differential effect when compared to other ligands for that receptor. In the vast majority of cases, such information is incomplete or missing. Thus, other approaches are needed to study and exploit biased agonism. Central to this effort is defining the relationship between receptor conformations stabilized by different agonists and their physiological effect. Such approaches are currently reductionist. One way to link the action of a ligand to its ability to stabilize distinct conformations of a receptor may be to use biophysical methods such as fluorescence spectroscopy[17]. However, such approaches are currently not sufficiently practical for large-scale GPCR drug-discovery programs and must be associated with more traditional measurements of receptor function. Thus, a practical compromise might be to use approaches such as the operational model of agonism for initial bias quantification and compound clustering, and then introduce an additional step to measure the binding kinetics of key representative compounds from each bias cluster. At the very least, this process can provide further insight as to whether there is a kinetic component to observations of bias and thus a more informed clustering of compounds for subsequent testing. This approach can also be used with biased allosteric modulators, although there is currently a paucity of labeled allosteric probes relative to orthosteric ligands. Nonetheless, this does not preclude the determination of the effects of allosteric modulators at different time points to ensure that the influence of kinetics is considered when designing and interpreting experiments that compare their actions at different pathways. Combining functional studies with both equilibrium and kinetic binding studies may also provide additional insights into the impact of kinetics on allosteric effects. Although these approaches add additional steps to any drug-discovery program aimed at exploiting biased agonism or allostery, they also provide a more informed platform with which to drive this process.

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Competing financial interests

The authors declare competing financial interests: details accompany the online version of the paper.

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