Why classical receptor theory, which ignores allostery, can effectively measure the strength of an allosteric effect underlying the ligand efficacy

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Abstract

Background and Purpose: The classical theory of receptor action has been used for decades as a powerful tool to estimate molecular determinants of ligand-induced receptor activation (i.e. affinity and efficacy) from experimentally observable biological responses. However, it is also a well-recognized fact that the receptor-binding and activation mechanisms, and the parameters thereof, described in the classical theory contradict with the modern view of receptor activation based on allosteric principles. Experimental Approach: We used mathematical analysis, along with some numerical simulations, to answer the key question as to what extent the classical theory is compatible -if at all- with the modern understanding of receptor activation. Key Results: Here, we showed conclusively that 1) receptor activation equations based on allosteric principles contain the logic of the classical theory in disguise, and therefore, 2) estimates of "intrinsic efficacy" (?) obtained by means of classical techniques (i.e. null methods or fitting the operational model to concentration-response data) are equivalent to the allosteric coupling factors that represent the molecular efficacy of ligands. Conclusion and Implications: Thus, we conclude that despite the right criticisms it has received so far, the classical theory may continue to be useful in estimating ligand efficacy from experimental data, if used properly. Here, we also provide rigorous criteria for the proper use of the theory. These findings not only have implications on ligand classification, but also resolve some long lasting discussions in the field of bias agonism in GPCR, which requires reasonable estimates of relative ligand efficacies at different signalling pathways.

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Running Title: Reconciling classical theory of receptor action with allostery

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Key Words: Quantitative receptor theory, Agonism, Efficacy, Allosterism, Cooperativity, GPCR, Cell signalling.

Introduction

Binding and effect, namely, recognizing a ligand at a specific binding site and changing activity state as a result, are fundamental tasks that receptors and many other functional proteins execute in performing their biological role. Revealing the mechanism that couples binding and activity in a protein is central to understanding function. This is especially true in receptor pharmacology, as the information on binding and activation are both encoded in the structure of the ligand. Thus, unlocking that code may help to design new molecules for drug discovery.

Not surprisingly, a quantitative theory that links ligand binding to receptor response was put forward in the early days of pharmacology (Ariens, 1954; Clark, 1933; Furchgott, 1966; Stephenson, 1956), when little was known about the molecular nature of receptors and the biochemical details of biological signal transduction. Nevertheless, the resulting conceptual framework, referred nowadays as classical theory of receptor action, has been most influential for the analysis of experimental concentration-response (CR) curves and for ligand classification in pharmacological studies. Its general power as both a conceptual and experimental tool is widely recognized also today.

In line with contemporary views of protein function, the headway to understanding the link between binding and effect in the receptor is the concept of allosteric regulation (Changeux, 2012; Weber, 1972; Wyman, 1967). However, the classical theory sharply contradicts the physicochemical principles on which allostery is based, both in the mechanism proposed for the definition of affinity and in the purported independence between affinity and efficacy (Colquhoun, 1987; Kenakin & Onaran, 2002).

Despite this contradiction, we previously noted (Onaran, Rajagopal, & Costa, 2014) a striking correspondence between “intrinsic efficacy” and the allosteric effect of an agonist on the receptor. Such a connection, however, was broken when efficacy estimation was based on an affinity value which was consonant with the allosteric theory (Onaran & Costa, 2021). This revealed an interesting paradox. The error in defining binding affinity, which renders the classical theory of pure historical value today, is also what makes it capable of recovering fairly accurate information about the relative magnitude of agonist’s allosteric effect. Originally we thought that such a coincidence was pure chance, but we were wrong.

Here we have analysed in depth the mathematical structure of the classical theory in comparison with the two possible schemes in which allosteric theory is translated into molecular models of receptor activation. We show that there is a common backdrop in classical receptor theory and allosteric models. In both, the relationship between ligand concentration and response takes the form of a composite function, where the inner one splits information about binding and the activation power of the agonist as separate and independent variables. This explains why intrinsic efficacy can capture the allosteric power of an agonist to put receptors in the active state, but also demonstrates that binding affinity in the classical theory should be considered as a 'virtual' entity, pretty much

equivalent to the concepts of “affinity for the reference state” (Colquhoun, 1987; Onaran & Costa, 1997; Onaran, Scheer, Cotecchia, & Costa, 2000), or “unconditional affinity” (Costa, Ogino, Munson, Onaran, & Rodbard, 1992; De Lean, Stadel, & Lefkowitz, 1980; Weber, 1972, 1975), which are central to the representation of allosteric coupling.

Results

Mechanism and logic of classical receptor theory

The classical theory is an occupational model developed collectively over decades with incremental refinements (Colquhoun, 2006b). Its essence can be summarized as follows: 1) The “*stimulus*”, an abstract quantity meant to express the aptitude of agonist-bound receptors to stimulate a biological response, is proportional to the amount of receptors occupied by the agonist and to the intrinsic efficacy (ε) of agonist that occupies the receptor. The parameter ε reveals the intrinsic capacity of the agonist to evoke a unit stimulus from a single receptor molecule. 2) The observed biological response is a nonlinear, but strictly monotonic function of this stimulus. The shape and the parameters of this function is determined solely by the cell system in which the receptor resides.

This scenario translates into the following formal statement:

ligand binding ([LR])	→	stimulus (s)	→	response (r)
$[\mathrm{LR}]=\frac{R_t[\mathrm{L}]}{[\mathrm{L}]+K_d}$	→	$s=\varepsilon[\mathrm{LR}]$	→	$r=f(s)$
	or	$f_1([\mathrm{L}])=\frac{\varepsilon R_t[\mathrm{L}]}{[\mathrm{L}]+K_d}$	→	$r=f_2\circ f_1([\mathrm{L}])\ ;\ f_2\equiv f$

..... Eq.1

Here, K_d is the equilibrium dissociation constant of the reversible binding reaction, [L]+[R]↔[LR] (with L and R standing for ligand and receptor) and R_t is the total concentration of receptor. This construct has following properties:

1. It explains commonly observed phenomena in pharmacological experiments, such as “receptor reserve” or changes in ligand behaviour from “full” to “partial” agonism among different tissues, even when ε and K_d are invariant.
2. It introduces two independent parameters ε and K_d that constitute the molecular determinants describing respectively the transducing and sensing elements of the ligand-receptor interaction. As such, these parameters depend on the identity of the two molecules (i.e. L and R) and do not change across cell systems, thus inspiring the idea that binding and effect may be independently profiled within the structures of ligand and receptor (Black, 1989).

3. With the monotonicity assumption of f, also known as the null principle, the theory provides experimental strategies to measure molecular properties of ligands from observed biological responses. All strategies for estimating from CR curves antagonist (Arunlakshana & Schild, 1959) and agonist (Furchgott, 1966) affinities or relative agonist efficacies (Barlow, Scott, & Stephenson, 1967) rely on this principle.

4. Replacement of the originally unspecified function $f(s)$ with a rectangular hyperbola (Black & Leff, 1983; Kenakin & Beek, 1982) or a logistic function (Black, Leff, Shankley, & Wood, 1985) yields the "operational" model, which is a regression equation meant to fit CR curves to obtain the same information as in point 3 above.

5. From a pure mathematical point of view, the structure of the theory can be seen as a composition of two nonlinear functions, namely a rectangular hyperbola describing s and the function $f(s)$ (eq.1). This feature of the theory leads to the main point of the present paper (See supplementary information S1 for details concerning points 3 to 5).

Despite the simple and yet elegant discrimination among molecule-invariant and cell-variant parameters in determining a biological response, which explains the enduring resonance of the theory in pharmacology, the assertion that ε and K_d are independent determinants of ligand action (point 2 above), attracted the fiercest criticism to the theory (Colquhoun, 1987, 1998). It can be summarised as follows: If as a result of ligand binding the receptor undergoes conformational/dynamical changes that make it capable of emitting a biological signal (e.g. the opening of ligand-gated ion channels), or enable it to interact with a transducer protein (e.g., as GPCRs do), those molecular changes must also affect the binding of the ligand. Therefore, ligand binding rules cannot be independent of what the ligand does to the receptor. If the mechanism is wrong, the molecular parameters that the theory defines are also wrong. However, as we demonstrate below, a detailed analysis of the structure of classical theory in comparison to allosteric equations shows otherwise.

Receptor mechanisms in allosteric theory

To model the change of the receptor's functional status caused by ligand binding, the receptor protein is postulated to adopt some "allosteric conformations" at equilibrium. By attributing some degree of functional activity to such allosteric states – e.g. 'active', 'active with respect to', 'partly active', 'inactive', etc., we envision receptor activation as the enhanced abundance of active states in the bound receptor form. In this formulation the notion of agonist efficacy finds a precise physical connotation; it represents the power of a ligand to populate with active states the conformational ensemble of the bound receptor, i.e. the ability of ligand to ease transitions towards a target state.

In an equivalent formulation of allosterism, allosteric transitions are not defined explicitly. Instead, they are implicit in the measurable correlation that exists between the free energy changes of ligand

binding interactions occurring at distinct sites of the protein. This is defined as cooperativity. As we have reviewed extensively before (Onaran & Costa, 2000, 2009, 2017; Onaran et al., 2014), there is no theoretical nor conceptual difference between the two formulation, and simple mathematical relations allow converting one formalism into the other. What seems to run the choice in receptor modelling is the molecular topography of the signal measured for assessing receptor activation. If that signal originates from an intrinsic protein activity, such as the conductance of an ion channel receptor, modelling based on allosteric states may be the simplest choice. However, if the receptor lacks intrinsic action in itself and it must interact with a transduction protein to initiate a biological signal –as it occurs in GPCRs- defining distributions of allosteric states that are jointly induced by both binding events (e.g. agonist and G protein binding) may be cumbersome, particularly if more than one active or inactive state appears to be involved in the process. In such cases, the formalism based on cooperativity provides a much simpler approach (Onaran & Costa, 2012).

In the next sections we examine two theoretical examples encompassing the alternative formulations of receptor activation according to allosteric theory. The first is a “state-transition” formulation. It describes a ligand-gated ion channel switching between a closed and an open state. Change of whole-cell ionic current, which reflects the output of a channel population, is measured in response to variation of agonist concentration. The second is a “cooperativity” formulation that describes a GPCR interacting with a transducer protein, where the concentration of receptor-transducer complex is measured in response to variable agonist concentration. In line with their theoretical equivalence, on comparing each alternative way of modelling allosteric theory with the logic of the classical receptor theory we reach the same conclusion.

State-transition formalism: simple ligand-gated ion channel

For analysing the difference between classical and allosteric models of receptor activation, we examine a whole-cell ionic current carried by an ensemble of identical channels, which is proportional to the average number (or concentration) of open channels. Although in real world most receptor channels display more than two states and bind more than one ligand molecule per receptor (Colquhoun, 2006a; Devillers-Thiéry et al., 1993; Sivilotti, 2010), here we use the simplest possible model to demonstrate the logic.

The average steady-state behaviour of such a simple two-state channel is fully described by the equilibrium scheme shown in fig 1.

Accordingly, receptor occupancy and response, as a function of ligand concentration [L], are given as follows (see supplementary information S2 for derivations):

$$Occupancy = \frac{R_t[\mathrm{L}]}{[\mathrm{L}] + K'} \quad ; \quad K' = \frac{1+j}{1+\beta j} K_d \; ; \; K_d = \frac{1}{k} \quad \text{..... Eq.2}$$

$$Net\ response = \frac{\eta\, R_t[\mathrm{L}]}{[\mathrm{L}] + K'} \quad ; \qquad \eta = \frac{(\beta - 1)\, j}{(1 + \beta j)(1 + j)} \quad \text{..... Eq.3}$$

Note that a channel receptor, depending on the value of j, may show some levels of activity in the absence of agonist. However, in the classical receptor theory such a constitutive activity is not an option. Therefore, Eq. 3 was derived to express only ligand-induced activity, i.e. "*Net response*" above the baseline current. Occupancy in eq.2 looks like a simple binding equation as in classical theory (eq.1), but the parameter K' (i.e. the effective affinity in the allosteric model) is a composite constant. It contains all constants of the system, including the 'efficacy' parameter β, and thus excludes the notion that agonist affinity and efficacy can be set apart in the mechanism of receptor activation.

The net response in eq.3 is proportional to occupancy, with a proportionality constant η that occupies in this equation the same position as ε does in the stimulus expression of the classical theory (eq. 1). However, η cannot be identified with the intrinsic efficacy ε, because it depends not only on β but also on j (i.e. basal equilibrium between open and closed states). Moreover, the quantity η is a nonlinear and saturable function of β, such that all high-efficacy agonists with increasingly larger β values maintain the same value of η (see fig.2). In fact, η is simply the maximum net response of an agonist.

In conclusion, the equations of the allosteric model seem to be inconsistent with the classical theory. However, a close inspection of eq. 3 reveals a pattern which is also present in the ligand/response relationship of classical receptor theory. In fact, we can show that the relationship between [L] and net response in the allosteric model is the result of a function composition, where two rectangular hyperbolae, one feeding the other, generates the relation shown in eq. 3. The two functions of this composition are as follows (see supplemental information S2 for derivation):

$$r = f_2(s) = E_m \frac{s}{s + B} \quad ; \quad E_m = R_t \frac{1}{1 + j} \quad ; \quad B = \frac{1 + j}{j} \quad \text{.....Eq.4a}$$

$$s = f_1([\mathrm{L}]) = (\beta - 1)\frac{[\mathrm{L}]}{[\mathrm{L}] + K_d} \quad \text{.....Eq.4b}$$

The function f_1 (Eq.4b) imitates the stimulus of the classical theory (eq.1), where ε is replaced by the efficacy term ($\beta - 1$), which is distinct and independent from the affinity parameter K_d. Thus, the identity $\varepsilon \equiv (\beta - 1)$ is automatically implied. However, K_d in this case is the ligand dissociation constant for the closed state of the receptor (eq. 2), but not the overall dissociation constant of the agonist-receptor complex. The function f_2 (Eq. 4a), which plays the role of the unspecified function $f(s)$ in the classical theory (eq.1), is a rectangular hyperbola on s, and contains only ligand-independent system parameters (i.e. R_t and j). Thus, the two-step logic of classical receptor theory is also present in the

allosteric description of receptor activation, although the variables defined by the pair of nested functions in the two models do not have the same meaning.

The composite function $r = f_2 \circ f_1([\mathrm{L}])$ in the allosteric model (i.e. Eqs. 4a and 4b) can also be expressed in the format of the operational model. In this case, the operational transduction coefficient τ, which typically blends the agonist-dependent (β) and system-dependent (j) terms of receptor action, is given as in Eq. 5 (see supplementary information S2 for derivation):

$$\tau = \frac{(\beta - 1)\, j}{1 + j} \equiv \frac{\varepsilon}{B} \quad \text{.....Eq.5}$$

Overall, the implication is that if we analysed CR curves for ligand-induced changes of whole cell current using the classical theory, either by data fitting with the operational model or by applying a null method, the relative ε values would directly give the relative allosteric effects that the ligands have in converting the receptor channel into an open form. For this to be true, however, we should use for computing ε the binding constant K_d for the closed state (Eq.4b), which is not an experimentally accessible constant (although it could be calculated from experimentally measured K', and the j and β values obtained from single-channel experiments). If we used the experimentally measurable K' (i.e., effective ligand affinity in Eqs. 2 and 3) for operational model fitting or null method analysis, the obtained values of relative ε or τ would yield an estimate of relative values of η (Eqs. 3) instead of the true allosteric efficacy β. (see S1 for the formal argument).

Obviously, applying classical receptor theory to ligand-operated channels has no relevance in experimental practice, since the value of β can be measured directly, at least in principle, from single-channel statistics observed in the absence and saturating presence of agonist. Yet, the channel example discussed here provides useful insight into the common mathematical and logical structure that is shared by classical theory and allosteric models of receptor activation. This common framework is also evident and becomes more relevant in the case of GPCR-like receptors, as exposed in the next section.

Cooperativity formalism: G protein-coupled receptor

Unlike channel receptors, GPCRs do not comprise any intramolecular reactivity that generates biological signals. Upon agonist binding these receptors must engage and activate external transducer proteins, such as G proteins and β-arrestins. Due to the complexity of the interactions between receptor, transducer molecules and the resulting events (e.g., nucleotide exchange on Gα, subunit dissociation in G protein, phosphorylation-dependent arrestin binding, etc.) any effort to realistically model the full mechanism of GPCR-mediated signalling in a cell is both difficult and useless. However, if the modelling is only meant to describe the allosteric change that makes a ligand-bound receptor more capable of interacting with a transducer protein T, the task is simpler,

as illustrated in the equilibrium scheme of Fig.3. The cooperativity factor α in fig.3, named free-energy coupling by Weber (Weber, 1972), represents the macroscopic effect that L binding adds to the apparent affinity of T, which is quantitatively identical to what T binding adds to the apparent affinity of L.

As discussed elsewhere (Onaran & Costa, 1997, 2017; Onaran et al., 2000), underneath the cooperativity factor α there may be a sum of allosteric factors that the two ligands L and T exert on all possible distributions of allosteric states of the receptor in transit from the free form R to the ternary bound form LRT. Unlike the channel case, however, these allosteric states cannot be experimentally accessed in GPCRs. Cooperativity, instead, is easily measurable. Evidently, the free energy change underlying α provides an allosteric representation of ligand's efficacy. A ligand with $\alpha > 1$ is an agonist, whereas ligands with $\alpha \leq 1$ are antagonists or inverse agonists.

For deriving equations describing receptor occupancy and response for the system shown in Fig.3 we follow the same procedure used for the channel case. We define as response a signal that is proportional to the amount of receptor complexed to T. In fact, the response thus defined has also a practical relevance in GPCR research, since it became almost a standard experimental approach nowadays to measure ligand-induced receptor-transducer binding by means of proximity assays (e.g. FRET or BRET) for assessing ligand action in GPCRs. Like in the channel case, we only evaluate “net response” by subtracting constitutive activity, which might be significant for large values of the binding constant m. However, while in the channel case receptor activation is described by first-order reactions (i.e., R ↔ R* and LR ↔ LR*), in the GPCR case receptor complexing to T are second-order reactions dependent on the free concentration of transducer, which changes with variation of ligand concentration. Therefore, we adopt a pseudo-first-order approximation that holds when total transducer concentration is much larger than that of total receptor (i.e., $T_t >> R_t$). Conditions outside of this restriction are evaluated in the next section using numerical simulations. With the above assumptions in place, occupancy and net response are respectively given by following equations (see supplementary information S3 for derivation):

$$Occupancy = \frac{R_t[\mathrm{L}]}{[\mathrm{L}] + K''} \quad ; \quad K'' = \frac{1 + m[\mathrm{T}]}{1 + \alpha m[\mathrm{T}]} K_d \; ; \; K_d = \frac{1}{k} \qquad \text{Eq.6}$$

$$Net\ response = \frac{\eta\, R_t[\mathrm{L}]}{[\mathrm{L}] + K''} \quad ; \quad \eta = \frac{(\alpha - 1) m[\mathrm{T}]}{(1 + \alpha m[\mathrm{T}])(1 + m[\mathrm{T}])} \qquad \text{Eq.7}$$

Here we call the effective affinity as K'' to distinguish it from that of the channel example. We should note, however, that while Eq. 6 is valid regardless of the pseudo first-order assumption (although K'' is not a constant in its absence), the functional form of the relationship describing net response (Eq. 7) changes when the condition $R_t << T_t$ is not valid (see S3 for more details). The formal similarity between the GPCR (Eqs. 6&7) and the channel (Eqs. 2&3) equations implies that the results of the

analysis made in the previous section for the state-transition formalism also apply to the cooperativity formalism: The net response (eq.7) can be broken down into two nested hyperbolae as shown below:

$$r = f_2(s) = E_m \frac{s}{s+B} \;;\; E_m = R_t \frac{1}{1+m[T]} \;\; \& \;\; B = \frac{1+m[T]}{m[T]} \quad \text{.....Eq.8a}$$

$$s = f_1([\mathrm{L}]) = (\alpha - 1)\frac{[\mathrm{L}]}{[\mathrm{L}]+K_d} \quad \text{.....Eq.8b}$$

Note that Eqs. 8a and 8b are identical to Eqs 4a and 4b, except that α in Eq.8b replaces β (Eq.4b) and the term $m[T]$ in Eq.8a replaces j (Eq.4a). The dissociation constant K_d, which in the channel case represents ligand's affinity for the closed state, in the GPCRs case denotes the unconditional affinity of the ligand for the receptor in the absence of T (i.e. 1/k in fig. 3). Thus, unlike the K_d, for the closed channel, which is not directly measurable, the unconditional K_d of GPCRs can be measured by performing ligand binding experiments on a receptor isolated from the transduction protein T. The linear relationship between allosteric effect of the ligand and Furchsgott's intrinsic efficacy ε also holds in the GPCR case, i.e $\varepsilon \equiv (\alpha - 1)$, indicating that such a relation is universally valid regardless of the formalism used to describe allosterism.

Relation between ε and α under non pseudo-first-order conditions

Are the conclusions drawn from the previous analysis extendible to different conditions (i.e., when $R_t \approx T_t$ or $R_t > T_t$)? To address the question, we performed several rounds of numerical simulations as described in the legend of fig.4. The results are summarized in Fig. 4.

Overall inspection of Fig.4 (lower panels) indicates that the intrinsic activity computed with the null method yields reasonably faithful estimates of the true allosteric efficacy of ligands under all receptor-transducer ratios studied ($R_t > T_t$, $R_t = T_t$ and $R_t < T_t$). When the receptor concentration falls below (Fig.4, lower left panel) or exceeds (Fig.4, lower right panel) the concentration of T_t, the theoretical lines overlap the scatter of $\Delta\varepsilon$ estimates. Notably, a more significant deviation is observed when $R_t = T_t$ (Fig.4, lower middle panel); even so, the deviations are limited to the partial agonism range and are rather small compared to experimental error usually encountered in such experiments. Also of note, the magnitude of basal activity (high and low m values for each condition) seems to have no significant effect on the accuracy of the relative efficacy estimates, as shown by the large overlap between black and grey dots in all plots, with only a minor tendency to spread apart when $R_t > T_t$ (Fig.4, lower left panel).

However, the scenario is radically different if as input for the null method calculations the effective affinity K'' is adopted instead of using the unconditional K_d values (Fig. 4, upper panels). In this case what is measured as efficacy is not the parameter (α -1) of the first function f_1 (Eq.8b), but the parameter η of the composite function shown in Eq.7. This parameter not only includes the agonist

dependent factor (α -1), but also the system-dependent parameters $[T]$ and m, and is not linearly related to the value of α. Moreover, as shown in Fig.4 (upper panels), the estimates of η are strongly dependent on the magnitude of constitutive activity dictated by m. Therefore, these simulations indicate the accuracy of intrinsic efficacy as a measure of the power of a ligand to allosterically activate the receptor is minimally impaired by violations of pseudo-first-order approximation. What plays a crucial role instead is the kind of affinity parameter that is used for determining the parameter ε.

Overall, the above analyses show that the right choice of affinity should be the closed-state affinity or the unconditional affinity in the allosteric or cooperative case, respectively. The latter choice is equivalent to fixing an appropriate ground-state for the receptor in a particular context, as schematized in Fig. 5.

Discussion

In this paper we show that allosteric/cooperative mechanisms of receptor activation formally contains Stephenson's historical construct in disguise as summarized schematically in Fig. 6.

For a long time the classical theory of receptor action has been the only established tool for gaining information about the molecular properties of ligand-receptor interaction from CR curves. What made this model so robust as to be useful even today is the logical separation of molecular intrinsic constants from locally dependent variables in the description of a ligand-elicited response. This is achieved through a mathematical structure based on the composition of two functions. A stimulus function s (f_1 in eq.1) depicting the molecular mechanism of ligand-induced receptor activation and the response function f_2 (eq.1) that specifies how the stimulus is revealed by the signalling network of a cell. This structure allows one to access the parameters of f_1 (i.e. ε or K_d) from the observed response (i.e $f_2 \circ f_1$) by means of null methods (or operational model fitting) of CR data.

However, the mechanism outlined in the stimulus function, which defines efficacy and binding affinity as uncorrelated constants, is wrong and inconsistent with protein physics. In fact, a ligand that changes the energy of receptor activation cannot do so without correspondingly altering binding energy. Despite this theoretical fault, a variety of experimental and numerical studies have shown that the determinations of ligand intrinsinc activity made according to classical theory can closely match the estimates of the relative allosteric effects of ligands obtained by applying biochemical models of allostery. Which poses the question of why the parameter ε, which is nowhere close to any physical definition of allosteric constants, can capture the strength of the allosteric effect of an agonist. In this paper we provide an answer to this question.

Here we show that the mechanism of receptor activation drawn according to allosteric theory (unlike the stimulus function in classical theory) is itself composed of two independent functions f_1 and f_2 (eqs 4 or 8). Here f_1 is not equivalent to the stimulus of the classical theory, but it represents the potential of a ligand to induce receptor activation, given its ability to stabilize active states (i.e., the "efficacy" parameters α or β) and the binding affinity for the ground state of the receptor. How this potential unfolds as receptor activation is specified by the second function f_2 that encloses ligand-independent molecular parameters, such as receptor density (R_t), the basal gating j (in the channel case), or the concentration [T] and the receptor affinity m of the transducer protein T (in the GPCR case). Thus, even when receptor activation is measured at the molecular level (as in the two examples we described above), the null method (or operational model fiting) of classical theory are still valid tools for estimating the ligand-dependent parameters of f_1 (i.e. α, β or K_d).

If the measured response is a biological signal distal to receptor activation, then the composite function representing allosteric activation must be joined by a third function describing the relation between activated receptor and recorded biological signal. The question is whether in this

composition of three functions (i.e. *response* = $f_3 \circ f_2 \circ f_1$) the methods of classical theory can still retrieve the molecular allosteric constants. Indeed, since both f_3 (the role of cell-dependent signal processing) and f_2 (the role of system-dependent molecular parameters) are completely ligand-independent, the composition $f_3 \circ f_2$ works effectively as the response function of the classical theory in converting the output of f_1 to biological effect. Hence, the two-step logical structure of classical theory is preserved and allows accessing the constants in f_1 (i.e. α, β or K_d). This explains why the intrinsic efficacy of classical model can provide an accurate estimate of the relative allosteric constants of ligands. It is important to note, however, that while the structure of biochemical allosteric models serendipitously works in making ε a valid measurement of the allosteric efficacy of agonists, the deliberate attempt of correcting the classical theory by replacing the stimulus expression (f_1) with an allosteric activation equation (i.e. eq.3 or 7) does not so. In fact, the parameter η of the allosteric equations cannot be identified with molecular efficacy, even though it occupies the same mathematical position as ε in the stimulus expression.

In summary, our analysis not only reconciles allosteric receptor activation with the classical theory as an estimation tool, but also provides a definite answer to long lasting discussions as to which affinity value should be used in efficacy estimation (Kenakin, 2014; Kenakin, Watson, Muniz-Medina, Christopoulos, & Novick, 2012; Kolb et al., 2022; Onaran & Costa, 2021): The agonist K_d should be the one for the inactive receptor state (closed channel or uncoupled receptor form), but not the effective one that governs overall receptor occupation.

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Figure legends

Fig.1 Equilibrium description of a two-state ligand-gated ion channel. R and R* signify closed and open states of the channel, respectively, whereas [R, R*] and [LR, LR*] represents two ligation states of the channel (unbound and bound, respectively). j is the equilibrium gating parameter, defined as [R*]/[R], k is the ligand affinity for the closed (ground) state defined as [LR]/[L][R], and β is the allosteric coupling constant identified with the molecular efficacy of the ligand, as it gauges the ligand effect on the gating equilibrium. β>1 implies that the ligand is an agonist which shifts the gating equilibrium towards the open state upon binding to the channel, at an extent depending on the magnitude of β. Note that the overall gating equilibrium is the sole determinant of the activity of the channel population observed via whole-cell ionic current, if the ionic composition, electric potential across the cell membrane and the number of channels on the membrane are kept constant during the experiment.

Fig.2 Response of a two-state allosteric system to agonists with different molecular efficacies. On the left panel, responses (or equivalently stimuli) simulated according to eq.3 are shown for 9 agonists that possess different β values. Log β values are indicated next to each curve in the picture. On the right panel, the proportionality constant η between occupancy and stimulus calculated according to eq.3 is shown depending on the (log) value of β. Correspondence between maxima and η are also shown with dotted lines. Parameter values common to all ligands are indicated in the picture. For the sake of simplicity, binding constant K_d for the ground state of the receptor is chosen equal for all ligands. Note that the value of η saturates with increasing β, and thus, cannot discriminate high efficacy ligands after a certain point (after log$\beta\approx$3 in this particular case). Moreover, the value of η is not proportional to β even for low efficacy ligands.

Fig.3 Equilibrium description of a cooperative system. R, L, and T signify receptor, ligand and transducer molecules, respectively. The equilibrium is fully described by two unconditional affinity constants k and m, and a coupling constant α. The unconditional affinities (defined in the usual way) govern the binding of one component to the receptor in the absence of the other. The coupling constant α gauges the effect of ligand binding on receptor-transducer affinity (or vice versa). In that sense it is identified with molecular ligand efficacy for a given receptor and a given transducer. α>1 implies that the ligand is an agonist that increases receptor affinity for the transducer. Occupancy corresponds to total concentration of ligand bound species, i.e. [LR]+[LRT], whereas total concentration of transducer-bound receptor, i.e. [RT]+[LRT] is identified with measured “activity”.

Fig.4. A Monte Carlo simulation for relative efficacy estimation in a cooperative activation scenario. Randomly generated agonist CR curves were analysed by means of a null method to estimate relative agonist efficacies at indicated conditions. Estimated relative values are plotted against relative α-1 values used in the simulations, on double-log scales. Agonist with highest efficacy (α) in each group representing a given condition, is chosen as the reference ligand. In the upper (blue scale symbols), and lower (grey scale symbols) panels effective (K’) or unconditional (K_d) binding constants were respectively used in the estimation procedures (indicated as η or ε in the picture). Red dotted lines indicate identity lines. T_t and R_t signify total transducer and receptor concentrations, respectively. Absolute value of total receptor is equal to 1 in all cases. Indicated (approximate) basal activities (i.e. 8% or 44%) were obtained by choosing different pairs of m values (i.e. unconditional R-T affinity) for indicated conditions as follows: 0.1, 1 (first column); 0.1, 1.4 (second column); 0.01, 0.08 (third column), respectively. **Simulation Strategy**: For each condition indicated in the picture (columns 1 to 3), agonist CR data were generated for 2000 agonist identified with their k and α values that were randomly picked from a log-uniform distribution in the ranges [-8,-5] and [0.3, 3.5], respectively. Data points (24 per

curve) were calculated by solving the equation for [RT]+[LRT] numerically (Pradines, Hasty, & Pakdaman, 2001). A Gaussian noise with a constant coefficient of variation (3%) was added to each data point. Each curve was then fitted with an empirical 4-parameter logistic equation and net responses were calculated by subtracting the corresponding basal activities in each curve. The entire set of fitted curves for a given condition was analysed by the null method of Barlow (Barlow et al., 1967). Calculations were done in Matlab (v. R2017a, MathWorks Inc).

Fig.5. A schematic representation of theoretically measurable ligand efficacies by invoking the classical theory. Ground and target states are schematized for different scenarios as indicated in the picture. In the allosteric and the cooperative cases, the ligand efficacies are identified with β and α, respectively, which dictates the extent at which the agonist favours the corresponding target states upon binding to the receptor. These efficacies are theoretically accessible by means of classical techniques, only if ligand affinities for the appropriate ground states are used in the estimation procedures. Distinction between the two arrows given for the cooperative case is only imaginary: In the first arrow, the ground state of the receptor (indicated by **r**) is imagined to be consisting of a mixture of allosteric states, whereas in the second arrow the same ground state (indicated by a blue font) is considered just to be the ligand-free receptor without any reference to its internal affairs. However, this distinction is absolutely inconsequential, since the resulting efficacies represent literarily the same final effect (i.e. α).

Fig.6. Correspondence between allosterism and classical theory of receptor action. Conceptual components of receptor signalling and their corresponding formulations in classical or allosteric theory are shown in the columns. Recognition and transduction are deemed independent in the classical theory whereas they are intricately convoluted in the allosteric/cooperative case. Nevertheless, the latter brakes down into virtual entities that perfectly complies with the formal structure of the classical theory, as indicated in the red box. The virtual response function in the allosteric/cooperative case is indicated as f' to emphasize its distinct meaning from the one intended in the classical theory (yellow shaded f), although both play the same mathematical role in the theory. Pure ligand-dependent parameters, i.e. K_d and ε (or α / β) are indicated in the picture wherever they are relevant. The parameters K', K'' and η that govern the composite function of occupancy+stimulus in allosteric/cooperative case are defined in eqs.3 & 7. Blue dotted arrows indicate estimation procedures by using null methods (or operational fit): These arrows stem from an observed response and point to the resulting estimate of an efficacy parameter (i.e. η or $\varepsilon \equiv \beta$-1 or α-1), depending on which affinity value is used in the corresponding procedure (indicated by black dotted arrows pointing to the relevant estimation procedure). Note that the presence of f' in the allosteric/cooperative case always necessitates a null method to estimate efficacy, even when the observed response is the receptor activation (stimulus) itself.

Figures

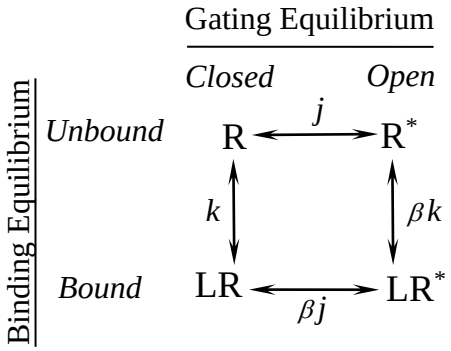

Fig.1

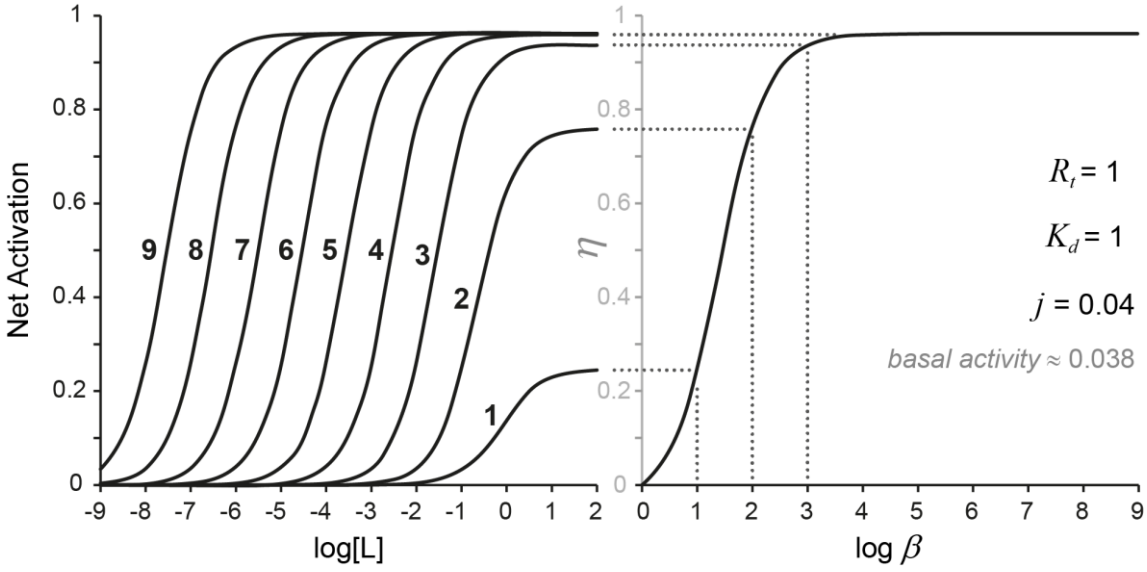

Fig.2

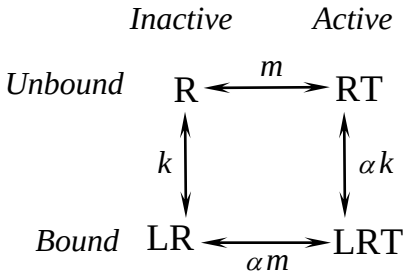

Fig.3

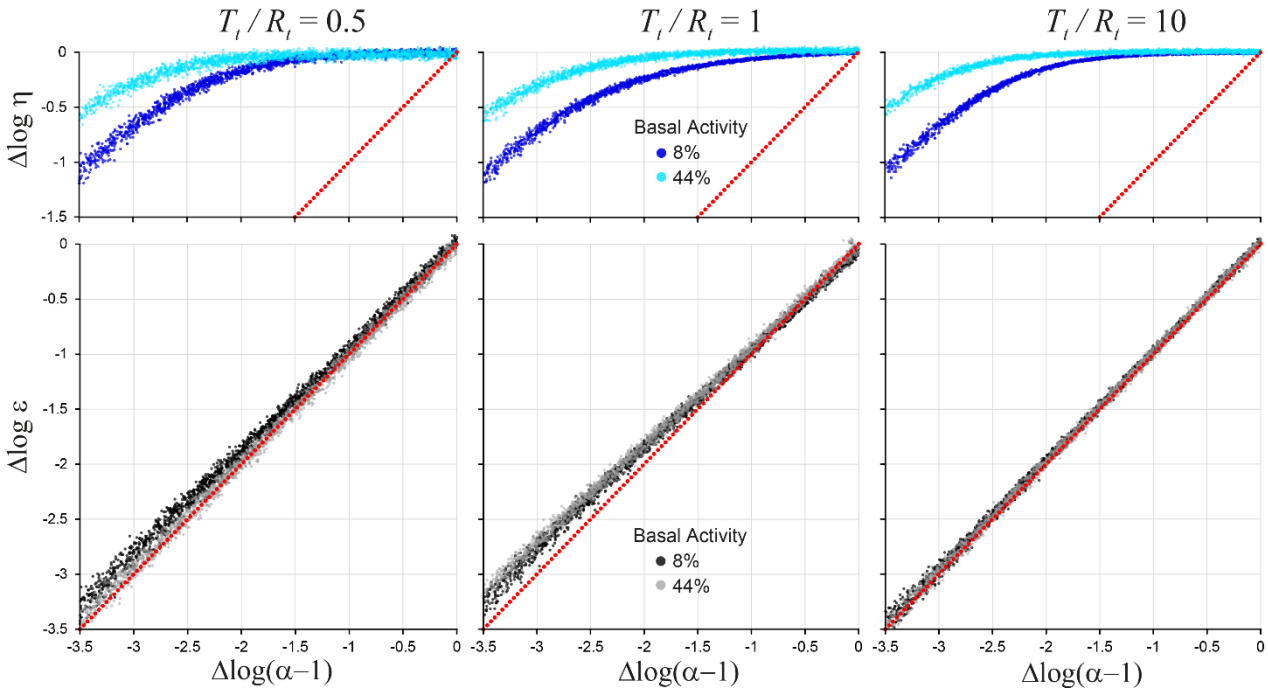
$T_t / R_t = 0.5$
$T_t / R_t = 1$
$T_t / R_t = 10$
Δlog η
Δlog ε
Basal Activity
8%
44%
Basal Activity
8%
44%
Δlog(α−1)
Δlog(α−1)
Δlog(α−1)

Fig.4

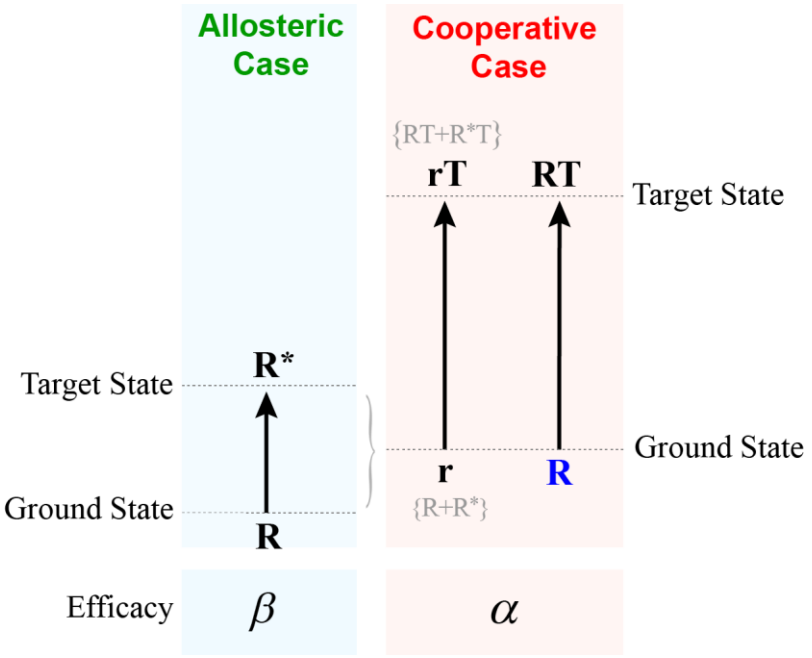
Allosteric Case
Cooperative Case
{RT+R*T}
rT
RT
Target State
R*
Target State
Ground State
r
R
Ground State
{R+R*}
R
Efficacy
β
α

Fig.5

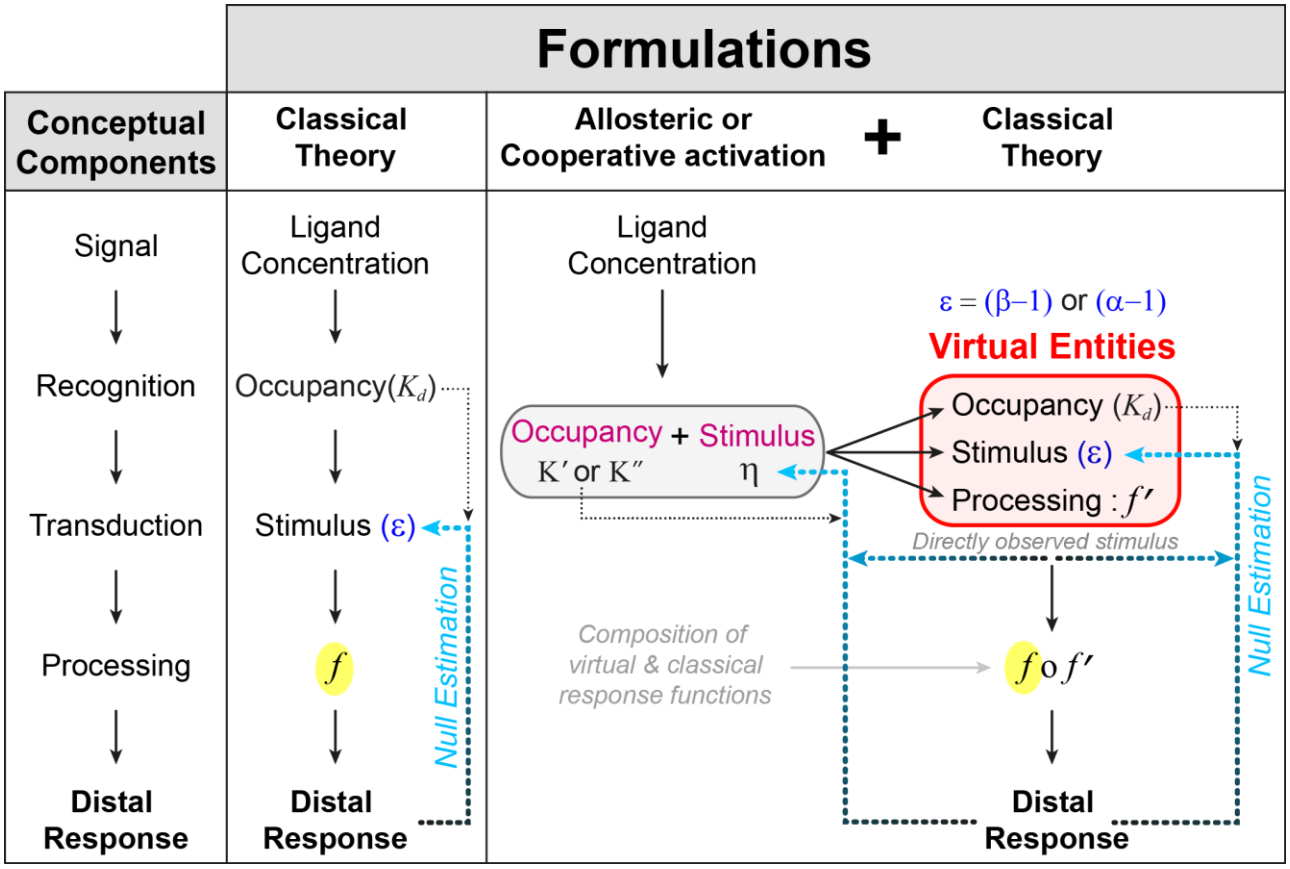
Formulations
Conceptual Components
Classical Theory
Allosteric or Cooperative activation
+
Classical Theory
Signal
Recognition
Transduction
Processing
Distal Response
Ligand Concentration
Occupancy(K_d)
Stimulus (ε)
f
Distal Response
Null Estimation
Ligand Concentration
ε = (β–1) or (α–1)
Virtual Entities
Occupancy + Stimulus
K′ or K″ η
Occupancy (K_d)
Stimulus (ε)
Processing : f′
Directly observed stimulus
Composition of virtual & classical response functions
f o f′
Distal Response
Null Estimation

Fig.6