Title

Cooperativity Has Empirical and Ultimate Levels of Explanation.

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pathways and subnetworks that are critical for cell physiology and diseases. One such pathway is the PI3K/Akt/mTOR pathway, which is critical for regulating cell growth, proliferation, apoptosis, and glucose metabolism, and is frequently dysregulated in cancer. On the other hand, we also take a systems biology approach to analyzing signaling networks. For instance, in collaboration with Heng Zhu and Jiang Qian's laboratories at JHU, we developed a strategy based on functional protein microarrays and bioinformatics to experimentally identify substrates for 289 unique human kinases. We further constructed a high-resolution map of phosphorylation networks that connects 230 kinases to 2591 *in vivo* phosphorylation sites in 652 substrates, providing global insights into kinase-mediated signaling pathways. In short, I could also say we work on whichever projects excite us most.

Tell us something about your work that is exciting for you right now

I am very excited about testing our new 'activity architecture' hypothesis. The assembly/disassembly and enzymatic activities of protein nanomachines underlie all cellular functions, and dysregulated nanomachines are the ultimate culprits in cancer. Knowing when and where these nanomachines are active is, therefore, critical to understanding the molecular drivers for normal cellular functions as well as for tumorigenesis, yet current efforts to characterize the molecular constituents of the cellular machinery overlook this critical dimension. We seek to establish a new conceptual framework to specifically understand the cellular organization of molecular activities. We hypothesize that cellular biochemical activities are spatially organized into an 'activity architecture' via the specific organization of active molecules and their regulatory partners. This activity architecture, together with the structural and mechanical architecture of the cell, encodes all the information needed to drive cellular function. We further hypothesize that perturbations to this activity architecture, even by a few dysregulated driver molecules, could lead to detrimental effects on cellular functions, such as loss of control over cell growth, division, and death. We are developing a new generation of biosensor and imaging technologies to characterize the activity architecture of the cell and examine the dysregulated activity architecture in cancer cells.

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Letter

Cooperativity Has Empirical and Ultimate Levels of Explanation

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Controversy over the meaning of pharmacological parameters often arises because of a lack of appreciation of different hierarchical levels of analysis. In a recent letter in *Trends in Pharmacological Sciences*, Zhang and Kavana [1] concluded that my two-state model for allosterism lacks cooperativity, even though Figures 5 and 6 in my review [2] illustrate examples of how the two-state model yields specific cooperativity values. Here, I explain how the two-state model (receptor-state analysis) gives rise to the cooperativity parameter (α) of the allosteric ternary complex model (receptor-population analysis).

Figure 1A shows the allosteric ternary complex model [3]. No states are illustrated, only receptor complexes (*R*, *DR*, *RA*, and *DRA*). The parameters K_1 and K_2 represent the observed affinity constants (reciprocal of the concentration of ligand required for half-maximal occupancy) of the orthosteric (*D*) and allosteric (*A*) ligands, and α, the cooperativity constant. Thus, αK_1 represents the observed affinity constant of *D* when the receptor population is saturated with the allosteric ligand, and αK_2, the observed affinity constant of *A* when the receptor population is saturated with the orthosteric ligand *D*.

In this population analysis, receptor activation is denoted by efficacy terms, (ε_D, ε_A, and ε_0), which represent the fractions of the populations of the *DR*, *RA*, and unoccupied receptor (*R*) complexes in the active state, respectively. The parameter, β_1, represents the scalar by which allosteric ligand *A* alters the efficacy of orthosteric ligand *D* ($\beta_1\varepsilon_D$, fraction of the population of DRB complexes in the active state). The product of the allosteric effects on affinity (α) and efficacy (β_1) is denoted by the parameter, γ_1 ($\gamma_1 = \alpha\beta_1$). This parameter is also equivalent to the ratio $\varepsilon_A/\varepsilon_0$ and, hence, is determined by the allosteric ligand and constitutive activity.

In Figure 1B, the allosteric ternary complex model is illustrated by four populations of receptors representing the unoccupied (*R*) and the three types of occupied receptor complex (*DR*, *RA*, and *DRA*). In this example, each population contains 1000 receptors, and the active and inactive receptor states are denoted by yellow and blue colors, respectively. Given that each receptor complex (e.g., *DR*) represents a mixture of structures, there is no real receptor species that has an observed affinity of K_1 or an activity of ε_D. Rather, these parameters represent the weighted average values of the receptor population.

If we turn up the zoom lens (Figure 1C), the ligand-binding sites on each state can be seen to isomerize concertedly as the receptor transitions between states. The affinities of ligands for these states of the receptor are designated in the two-state

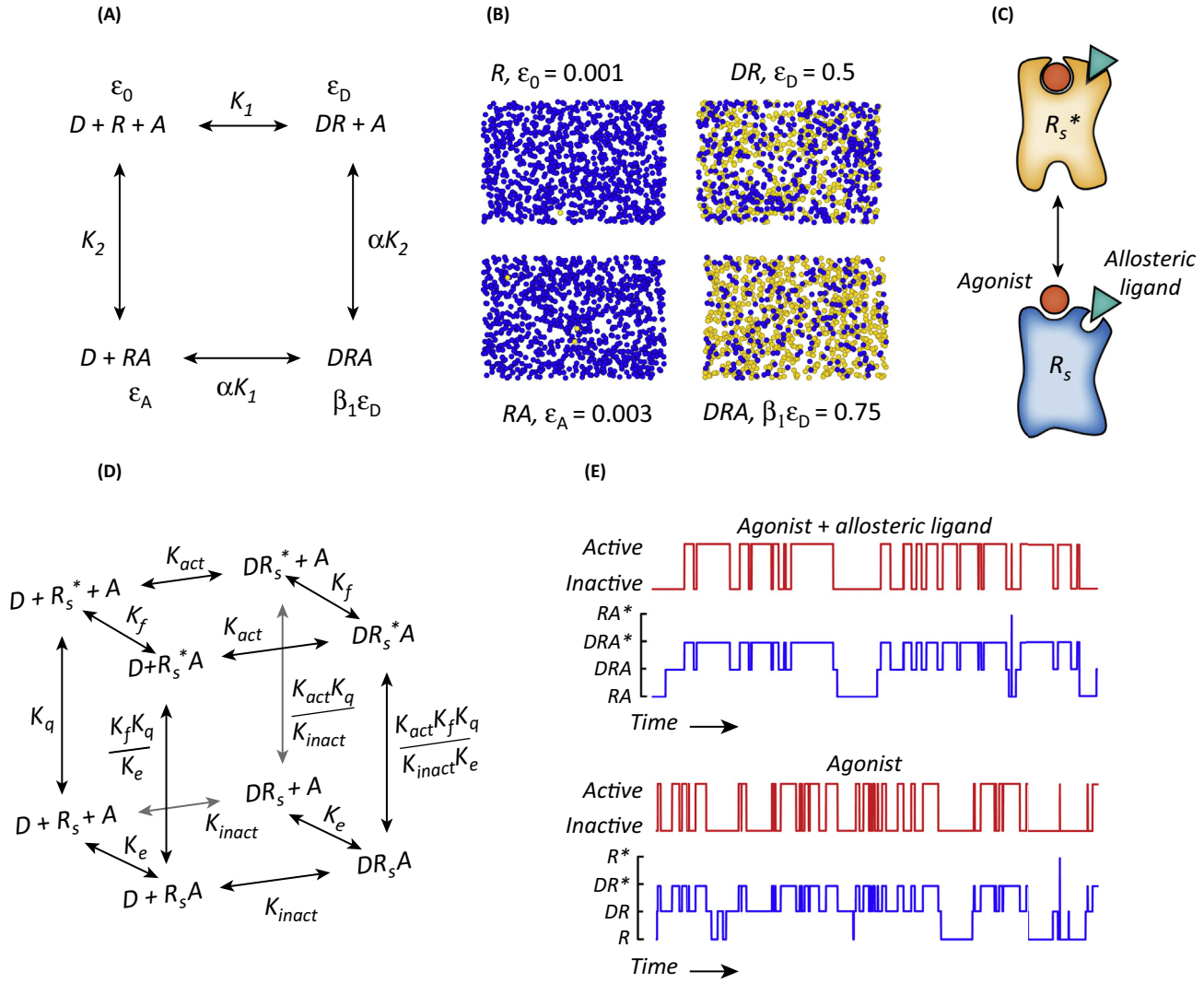

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Figure 1. Two Different but Consistent Ways of Quantifying Cooperativity Based on the Aggregate Receptor Population (Population Analysis) or Individual Receptors (State Analysis) as the Unit of Analysis. (A) This allosteric ternary complex model addresses the observed affinity of orthosteric (*D*) and allosteric (*A*) ligands for the receptor population (K_1 and K_2, respectively). These constants have inverse molar units and are defined accordingly (i.e., M^{-1}, K_1 = [*DR*]/[*D*][*R*]). The cooperativity constant, α, represents the scalar change in the observed affinity of each ligand caused by the binding of the other ligand to the receptor complex. This constant (α = 3.0 in this example) represents the measure of cooperativity at the population level of analysis. The fractional amount of the population of each type of receptor complex in the active state is denoted by an efficacy term (i.e., ε_0, ε_D, ε_A, and $\beta_1\varepsilon_D$ for *R*, *DR*, *RA*, and *DRA*, respectively). (B) This illustration is intended to represent the receptor population (1000 receptors) at an instant in time under four conditions: in the absence of ligand (*R*); in the presence of receptor-saturating concentrations of *D* (*DR*); *A* (*RA*); and both *D* and *A* (*DRA*). Active and inactive receptors are denoted by yellow and blue symbols, respectively. During this snapshot, the unoccupied receptor population (*R*) contains a single constitutively active receptor near the middle of its lower border and the allosteric ligand-occupied receptor population (*RA*) contains three, two near the center and one in the upper left quadrant. The active receptors in the *DR* and *DRA* populations are obvious. (C) A view of a single receptor isomerizing between active (R_s*) and inactive states (R_s). The structures of the two states are sufficiently distinctive such that the active state has the capacity to catalyze a signal, whereas the inactive does not. Both ligands (*D* and *A*) have characteristic affinity constants (microscopic constants) for the active and inactive receptor states. (D) The two-state model for allosteric interactions. Unlike the allosteric ternary complex model, the affinities of ligands are determined by the state or structure of the binding pocket regardless of whether one or two ligands are bound to the receptor complex. Cooperativity is determined by the selectivity of the ligands for the active state (i.e., K_{act}/K_{inact} and K_f/K_e for *D* and *A*, respectively). For this example of positive cooperativity (K_{act}/K_{inact} = 1000 and K_f/K_e = 3.0), both ligands have the effect of increasing the isomerization constant of the receptor (K_q). Thus, the cooperative effect of the allosteric ligand on the binding of the orthosteric ligand is equivalent to K_f/K_e (3.0). (E) The two-state model can be used to simulate single receptor activity as a continuous Markov process in the absence and presence of allosteric modulator. The transitions between the various types of receptor complex are indicated in blue, whereas the activity of the receptor is indicated in red. Here the positive cooperative effect of

(Figure legend continued on the bottom of the next page.)

Table 1. Equations Describing the Values of the Superficial Population Parameters in terms of the More Fundamental Receptor-State Parameters

Population Parameter	Function in terms of Receptor-State Parameters
K_1, *observed affinity constant of orthosteric ligand, D*	$K_1 = \frac{K_{inact}+K_{act}K_q}{1+K_q}$
K_2, observed affinity constant of allosteric ligand, *A*	$K_2 = \frac{K_e+K_fK_q}{1+K_q}$
α, cooperativity constant	$\alpha = \frac{(1+K_q)(K_{inact}+K_e+K_{act}K_fK_q)}{(K_e+K_fK_q)(K_{inact}+K_{act}K_q)}$
β_1, scalar effect of *A* on ε_D	$\beta_1 = \frac{K_{inact}K_f+K_{act}K_fK_q}{K_{inact}K_e+K_{act}K_fK_q}$
γ_1, product of α and β_1	$\gamma_1 = \frac{K_f+K_fK_q}{K_e+K_fK_q}$
ε_0, efficacy of the unoccupied receptor	$\varepsilon_0 = \frac{K_q}{1+K_q}$
ε_D, efficacy of the *DR* complex	$\varepsilon_D = \frac{1}{1+\frac{K_{inact}}{K_{act}K_q}}$
ε_A, efficacy of the *RA* complex	$\varepsilon_A = \frac{1}{1+\frac{K_e}{K_fK_q}}$

model shown in Figure 5A of my prior review [2], which is shown here in a modified format (Figure 1D). This model (simplified Monod-Wyman-Changeaux Model [4]) separates the mixed population of receptor complexes into active (R_s^*) and inactive (R_s) states, each designated with the subscript 's'. Given that the ligand-binding sites on the inactive receptor state have specific structures, each type of inactive-state receptor complex has the same affinity for a specific ligand. In other words, the affinity of *D* for R_s and R_sA is the same (K_{inact}) as is the affinity of *A* for R_s and DR_s (K_e). The analogous situation applies to the active state: the affinity of *D* for R_s^* and R_s^*A is the same (K_{act}) as is the affinity of *A* for R_s^* and DR_s^* (K_f).

Thus, the cooperativity of the more empirical allosteric ternary complex model (Figure 1A) does not arise because simultaneously bound allosteric and orthosteric ligands somehow change the structure of their respective sites by a scalar amount corresponding to the value of α, but rather, through a process of conformational induction. That is, the preferential binding of the positive allosteric ligand to the active state (i.e., $K_f/K_e = 3.0$) shifts the equilibrium between active and inactive states in the direction of the active state, causing an increase in the observed affinity of the agonist (Figure 1D). The vertical transitions show how the agonist, the allosteric ligand, and the combination of both ligands increase the isomerization constant (K_q) by factors of K_{act}/K_{inact}, K_f/K_e, and $K_{act}K_f/K_{inact}K_e$, respectively. This is the ultimate mechanism of cooperativity in the two-state model. How the fundamental state parameters give rise to the more superficial population parameters is described in Table 1 [5,6]. These equations differ from those given by Zhang and Kavana [1].

The two-state model can also be used to describe the behavior of a single receptor as it transitions through specific states [7]. The forward and reverse rate constants that define the receptor-state affinity constants can be used to calculate conditional probabilities for transitioning from a given state to adjacent states over a small time increment. The process is repeated iteratively to simulate the random behavior of the receptor in time (continuous Markov process). Figure 1E shows examples of single receptor behavior in the absence and presence of an allosteric modulator for the model shown in Figure 1D. It is impossible to use the population model (Figure 1A) for Markov analysis because there are no real receptor structures that correspond to the various receptor complexes in the model.

Presumably, the structure of the active state of the binding pocket of a GPCR when bound with a highly efficacious natural ligand is well defined, as are the more proximal sections of the helices that form it. However, there may be substantial movement of the cytosolic ends of the helices if there is no receptor-bound G protein to stabilize them. Similarly, the cytosolic ends of the helices may undergo movement in the inactive state even though the binding pocket may be stabilized. The two-state model is not based on the assumption of rigid receptor-state structures, but rather, on: (i) a capacity to induce a signal (active state) or not (inactive state); and (ii) specific binding-pocket structures with characteristic affinities. Of course, certain biased ligands can stabilize additional active states, and the latter can be included in a more complete multi-state model, as previously described [8].

I have not expanded the allosteric ternary complex model (Figure 1A) into active and inactive receptor states as Hall [9] and others [1] have because the efficacy terms already account for these. Also, the use of observed affinities and cooperativity constants (α and δ [9]) in these models implies additional undefined receptor states. The possible outcomes of these models greatly exceed those of receptor-state models and include behavior that is impossible if we assume that conformational induction is the engine that drives conformational change.

the allosteric modulator is manifest as an increase in both (i) receptor occupancy at low agonist concentrations; and (ii) the mean activation time of occupied receptors. The probability that a given type of ligand–receptor complex (e.g., $DR_s^* + DR_s$) is in the active state (DR^*_s) is equivalent to the time spent in the active state divided by the total time that both R_s and R_s^* are occupied by *D*. This probability is equivalent to the corresponding efficacy value given in (A) and (B) (i.e., ε_D).

For example, the expanded allosteric ternary-complex population model [9] includes in its suite of outcomes the case of two ligands that bind to their respective sites on a receptor and have no effect on receptor isomerization by themselves, but together cause substantial receptor activation (co-agonism). A problem with this mechanism has been described previously, as well as a more realistic explanation of co-agonism based on two receptor states [5].

A seemingly harmless property of the allosteric ternary complex model is that it fits data for the case of an allosteric ligand that always modifies only the affinity of orthosteric ligands. While it is possible to describe a four-state model that can explain affinity modulation without a change in efficacy, it is nearly impossible to use the model to explain the affinity-only modulation of a diverse group of ligands acting at the same receptor [5]. In the case of a putative allosteric modulator that exhibits only negative affinity modulation (e.g., gallamine), perhaps its binding pocket is so close to the orthosteric site [10] that one of its vibrating chemical moieties intrudes into the orthosteric-binding pocket when unoccupied and impedes the association of orthosteric ligands, thereby reducing their observed affinity.

A more extreme form of this latter idea might explain why the muscarinic antagonist, atropine, causes a maximal 10 000-fold shift in the concentration-response curve of the ectopic agonist, AC-42 {4-n-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine}, in a manner consistent with the prototypical limiting effect of allosteric antagonism [11]. Given the structure of AC-42, it might behave as a bivalent ligand that derives considerable affinity through the interaction of its 1-(1-oxobutyl)-2-methylbenzene moiety with an ectopic site on the muscarinic receptor, while its 4-*n*-butylpiperidine residue interacts with the orthosteric site to compete with atropine. Such an inhibitory mechanism is consistent with the behavior of the allosteric ternary complex model [12]. A consideration of the two-state model provides predictions regarding the efficacy of AC-42 that could be used to distinguish between allosteric and bivalent ligand mechanisms.

Expanded population models [1,9] seem to be favored because of their ability to describe a variety of experimental observations and to account for an unlimited number of receptor states. My view is more in line with that of Lander [13], who suggests that the value of models is not so much in their ability to describe data so much as how they help us to understand biology. The demonstration of a lack of consistency of functional data with a simple two-state model is useful because it helps us understand how drugs interact with receptors. It gives us a reason to consider additional defined states or perhaps to consider fundamentally different models.

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Forum

Controversy in Purchasing Prescription Drugs Online in China

Peng Yuan,[1] Lin Qi,[1] and Long Wang[1,*]

China's government is considering legalization of online prescription drugs to increase the pharmaceutical market and enhance access to necessary medicines. However, challenges such as a shortage of licensed pharmacists and drug quality issues have raised concerns and delayed consensus on the proposal. China's government must address the most pressing issues so it can render a decision on online prescription sales.

Controversy in Purchasing Prescription Drugs Online in China

China is experiencing accelerated development in the era of 'Internet plus medicine', which indicates an integration of the Internet and the pharmaceutical industry. A promising and booming pharmaceutical market combined with the Internet has been witnessed in recent years. In 2014, China's government came up with a new plan to allow online sales of prescription drugs with a legal prescription from a doctor. However, this plan has not reached