Evaluation of Operational Models of Agonism and Allosterism at Receptors with Multiple Orthosteric Binding Sites

Karen J. Gregory, Jesús Giraldo, Jiayin Diao, Arthur Christopoulos, and Katie Leach

Drug Discovery Biology and Department of Pharmacology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Melbourne, Australia (K.J.G., J.D., A.C., K.L.); Laboratory of Molecular Neuropharmacology and Bioinformatics, Institut de Neurociències and Unitat de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, Barcelona, Spain (J.G.); Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red de Salud Mental, Bellaterra, Spain (J.G.); and Unitat de Neurociència Translacional, Parc Taulí Hospital Universitari, Institut d’Investigació i Innovació Parc Taulí and Institut de Neurociències, Universitat Autònoma de Barcelona, Bellaterra, Spain (J.G.)

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ABSTRACT

Current operational models of agonism and allosterism quantify ligand actions at receptors where agonist concentration-response relationships are nonhyperbolic by introduction of a transducer slope that relates receptor occupancy to response. However, for some receptors nonhyperbolic concentration-response relationships arise from multiple endogenous agonist molecules binding to a receptor in a cooperative manner. Thus, we developed operational models of agonism in systems with cooperative agonist binding and evaluated the models by simulating data describing agonist effects. The models were validated by analyzing experimental data demonstrating the effects of agonists and allosteric modulators at receptors where agonist binding follows hyperbolic (M₄ muscarinic acetylcholine receptors) or nonhyperbolic relationships (metabotropic glutamate receptor 5 and calcium-sensing receptor). For hyperbolic agonist concentration-response relationships, no differences in estimates of ligand affinity, efficacy, or cooperativity were observed when the slope was assigned to either a transducer slope or agonist binding slope. In contrast, for receptors with nonhyperbolic agonist concentration-response relationships, estimates of ligand affinity, efficacy, or cooperativity varied depending on the assignment of the slope. The extent of this variation depended on the magnitude of the slope value and agonist efficacy, and for allosteric modulators on the magnitude of cooperativity. The modified operational models described herein are well suited to analyzing agonist and modulator interactions at receptors that bind multiple orthosteric agonists in a cooperative manner. Accounting for cooperative agonist binding is essential to accurately quantify agonist and drug actions.

SIGNIFICANCE STATEMENT

Some orthosteric agonists bind to multiple sites on a receptor, but current analytical methods to characterize such interactions are limited. Herein, we develop and validate operational models of agonism and allosterism for receptors with multiple orthosteric binding sites, and demonstrate that such models are essential to accurately quantify agonist and drug actions. These findings have important implications for the discovery and development of drugs targeting receptors such as the calcium-sensing receptor, which binds at least five calcium ions.
which is a function of both tissue- and agonist-specific components; it is the ratio of the total receptor number and a transducer parameter that defines the avidity with which a given agonist-occupied receptor complex promotes the final observed pharmacological effect. As such, the operational model of agonism is a useful tool for quantifying agonism in a comparable manner across different test systems (Black and Leff, 1983), and has subsequently been extended or modified to also quantify effects of allosteric modulators and biased agonists (Leach et al., 2007, 2010; Kenakin, 2012).

The operational model of agonism has been most commonly applied to characterize the activity of agonists that display both rectangular hyperbolic or nonhyperbolic concentration-response curves, i.e., normally empirically characterized by Hill slopes that are equal to or different from unity, respectively. The key underlying assumption in the majority of instances to date where an agonist concentration-response curve displays a Hill slope significantly different from 1 has been ascribed in the most common form of the operational model to differences in the postreceptor machinery that transduces occupancy to response, i.e., through introduction of a so-called transducer slope (n) (Black et al., 1985). For instance, steep or shallow Hill slopes could arise due to changes in the sensitivity of one or more steps in a receptor’s signal transduction mechanism, while the initial agonist-receptor binding event is assumed to be characterized by a simple hyperbolic one-to-one relationship. However, for ion channels and a number of G protein–coupled receptors (GPCRs), particularly the class C GPCR subfamily, nonhyperbolic concentration-response relationships can also arise from cooperative binding of multiple equivalents of the same endogenous agonist molecule prior to any subsequent processing of the stimulus by the cellular transduction machinery. For example, while a number of small molecule calcium-sensing receptor (CaSR) agonists produce responses characterized by Hill slopes close to unity (Cook et al., 2015; Keller et al., 2018), indicating a transducer slope equal to unity, it is also well established that CaSR responses to its endogenous activator, extracellular calcium (Ca\(^{2+}\)), and other divergent cations are characterized by extremely high Hill slopes, ranging from 2 to 4 (Brown, 1983; Davey et al., 2012; Leach et al., 2015). The most parsimonious explanation to account for these disparate observations is that the operational transducer slope linking CaSR agonist occupancy to response can adequately be described by a transducer slope equal to unity, which suggests that the cooperativity observed in response to activators such as Ca\(^{2+}\) ions arises at the level of binding, not function. This is also in accord with known pharmacological and structural studies of the CaSR that have identified multiple binding sites for Ca\(^{2+}\) ions (Geng et al., 2016). As a consequence, the classic operational model of agonism as applied to concentration-response curves of nonunit Hill slopes is suboptimal for such situations.

Herein, we sought to develop and evaluate an operational model of agonism that describes orthosteric agonist binding to multiple sites in a cooperative manner, referred to as the cooperative agonist operational model. The cooperative agonist operational model was superior to the original Black-Leff operational model of agonism in fitting Ca\(^{2+}\)-CaSR concentration-response data. We also extended this cooperative agonist operational model to incorporate allosteric modulation of the affinity and efficacy of an agonist that binds cooperatively to multiple sites. This operational model of allosterism with cooperative agonist binding was fitted to data describing the actions of CaSR positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), and revealed that if cooperative agonist binding is not taken into consideration, under- or overestimates of PAM and NAM affinity and cooperativity can occur.

**Materials and Methods**

**Materials.** Dulbecco’s modified Eagle’s medium (DMEM), Flp-In human embryonic kidney (HEK) T-REx cells, blasticidin S HCl, and FBS were obtained from Invitrogen (Carlsbad, CA), while hygromycin B was obtained from Roche (Mannheim, Germany). Fluoro-8 AM was obtained from Abcam (Cambridge, MA).

**CaSR-Expressing HEK293 Cell Lines.** The generation of DNA and Flp-In HEK T-REx cells stably expressing c-myc-tagged wild-type (WT) CaSR in pcDNA5/FRT/TO have been described previously (Davey et al., 2012; Leach et al., 2016). Flp-In HEK T-REx CaSR cells were maintained in DMEM containing 5% FBS, 200 μg/ml hygromycin B, and 5 μg/ml blasticidin S HCl. To generate a tetracycline-inducible Flp-In HEK cell line stably expressing an N-terminally truncated CaSR, N-terminally truncated CaSR corresponding to amino acids 600–903 with an N-terminal influenza hemagglutinin signal peptide followed by a c-myc epitope and rhodopsin signal peptide in pcDNA3.1+ (Leach et al., 2016) was transferred to pcDNA5/FRT/TO using BamHI and NotI restriction sites. Flp-In HEK T-REx cells were seeded into 25 cm² flasks in DMEM containing 5% FBS and allowed to reach 80% confluency. Cells were transfected with 0.5 μg pcDNA5/FRT/TO containing the N-terminally truncated CaSR plus 5 μg FOG44 with lipofectamine 2000 (1:1 DNA:lipofectamine 2000) according to the manufacturer’s instructions. The following day, cells were transferred to a T75 cm² flask, and 24 hours later DMEM was replaced with DMEM containing 5% FBS, 200 μg/ml hygromycin, and 5 μg/ml blasticidin S HCl. The selection DMEM was replaced every 3 days until untransfected cells had died (~10 days), and antibiotic-resistant cells were expanded and maintained in DMEM containing 5% FBS, 200 μg/ml hygromycin, and 5 μg/ml blasticidin S HCl. All cell lines were routinely tested for mycoplasma contamination using the Lonza MycoAlert mycoplasma detection kit.

**Determination of WT and N-Terminaly Truncated CaSR Cell Surface Expression Using Fluorescence-Activated Cytometry.** Flp-In HEK T-REx WT and N-terminally truncated CaSR-expressing cells were seeded at 80,000 cells per well into a 96-well plate and expression was induced with 100 ng/ml tetracycline overnight at 37°C. The following day, cells were harvested and washed in 1X PBS with 0.1% bovine serum albumin and 2 mM EDTA (wash buffer) before centrifugation (350g, 4°C for 3 minutes) before resuspension and 30-minute incubation in 100 μl blocking buffer (1X PBS, 5% bovine serum albumin, and 2 mM EDTA) containing 1 μg/ml AP647-conjugated 9E10 made in-house as previously described (Cook et al., 2015). Cells were washed as previously described and resuspended in wash buffer containing propidium iodide. Live cell fluorescence was measured using a BD FACS Canto analyzer (Becton Dickinson).

**Calcium Mobilization Assays.** Cells were seeded in clear 96-well plates coated with poly-D-lysine (50 μg/ml) at 80,000 cells per well and incubated overnight in the presence of 0 or 100 ng/ml tetracycline. The following day, cells were washed with assay buffer (150 mM NaCl, 2.6 mM KCl, 1.18 mM MgCl₂, 10 mM D-Glucose, 10 mM HEPES, 0.1 mM CaCl₂, 0.5% bovine serum albumin, and 4 mM probenecid at pH 7.4) and loaded with Fluoro-8 AM (1 μM in assay buffer) for 1 hour at 37°C. Cells were washed with assay buffer prior to the addition of fresh assay buffer.

For all studies, each well was treated with a single agonist concentration. The release of intracellular calcium (Ca\(^{2+}\)) was measured at 37°C using FlexStation 1 or 3 (Molecular Devices, Sunnyvale, CA). Fluorescence was detected for 60 seconds at 490 nm excitation and 520 nm emission and the peak Ca\(^{2+}\) measured at ASPET Journals on October 21, 2023 molpharm.aspetjournals.org Downloaded from
where \( A \) is the agonist concentration, dissociation constant; \( K_A \) is the equilibrium dissociation constant of the orthosteric agonist, which was fixed in some instances to the affinity determined in radioligand binding assays (Mutel et al., 2000; Leach et al., 2010); \( K_B \) is the equilibrium dissociation constant of the allosteric ligand; \( \theta_A \) and \( \theta_B \) are the operational efficacies of the orthosteric agonist and allosteric ligand, respectively; \( \alpha \) and \( \beta \) are the allosteric effects on orthosteric agonist affinity and efficacy, respectively (it should be noted that \( \beta \) is not a reciprocal efficacy cooperativity factor) (Leach et al., 2007; Giraldo, 2015); and \( [A] \) and \( [B] \) are the orthosteric agonist and allosteric ligand concentrations, respectively.

To fit the operational model of allosterism to data describing the interaction between Ca^2+ and cinacalcet at the CaSR, the original operational model of allosterism shown by eq. 3 was simplified, because for a full agonist like Ca^2+ (i.e., one that generates the maximal system response at submaximal receptor occupancies) \( K_A >> [A] \). Furthermore, because the CaSR’s orthosteric agonist, Ca^2+, was present in the assay buffer, the contaminating agonist was included in the equations used to analyze CaSR PAM (cinacalcet) and NAM (2-chloro-6-[(2R)-2-hydroxy-3-[2-methyl-1-naphthalen-2-ylpropan-2-yl]aminomethylene]benzonitrile [NPS2143]) data (Keller et al., 2018). Therefore, data describing the interaction between Ca^2+ and cinacalcet or NPS2143 at the CaSR were fitted to the original operational model of allosterism with the contaminating agonist (eqs. 5 and 6, respectively) or to an operational model of allosterism with cooperative agonist binding and contaminating agonist (eqs. 7 and 8, respectively):

\[
\frac{E_m([A + \theta_A [K_B + \alpha [B]]) + \theta_B [EC_{50}]^*]}{[EC_{50}]^* ([K_B + [B])]^* + ([A + \theta_A [K_B + \alpha [B]]) + \theta_B [EC_{50}]^*)} \quad \text{(5)}
\]

where \( EC_{50} \) is the agonist concentration that elicits a half-maximal response, in which it should be noted that inclusion of \( EC_{50} \) involves some simplifying assumptions that facilitate data fitting (Aurelio et al., 2009; Giraldo, 2015); \( C \) is the contaminating agonist concentration; and all other parameters are as described for eq. 3.

The Eqs. 1 or 2 were fitted to agonist concentration response data in order to quantify agonist affinity and efficacy. When fitting eq. 2 to experimental data, the transducer slope was constrained to unity (see Results for validation of this assumption).
where $[A]$ is the agonist concentration; and all other parameters are as described for eq. 4.

The Hill equation (Eq 9) was fitted to simulated data:

\[
\text{Effect} = \frac{E_{\text{max}} [A^{n_B}] 
\alpha + C_B^{n_B} (K_B + \alpha [B]) + \tau_B [B] [K_A^{n_B}]^\tau_B
\left([A + C_B^{n_B} K_B + K_B^{n_B} [B] + \alpha [A + C_B^{n_B} [B]]]^\tau_B + [\tau_B [A]^{n_B} (K_B + \alpha [B]) + \tau_B [B] [K_A^{n_B}]^\tau_B
\right)}{([A + C_B^{n_B} K_B + K_B^{n_B} [B] + \alpha [A + C_B^{n_B} [B]]] + \tau_B [A]^{n_B} (K_B + \alpha [B]) + \tau_B [B] [K_A^{n_B}]^\tau_B
\left)}
\]

where all other parameters are as described for eq. 4.

The Hill equation (Eq 9) was fitted to simulated data:

\[
\text{Effect} = \frac{[A]^{n_B} E_{\text{max}}}{[A]^{n_B} + EC_{50}^{n_B}}
\]

where $[A]$ is the agonist concentration; $E_{\text{max}}$ is the maximum agonist effect; and $n_B$ is the Hill slope.

Nonlinear regression analysis was performed in GraphPad Prism 7 or 8. Potency, affinity, cooperativity, and efficacy parameters were estimated as logarithms (Christopoulos, 1998). An extra sum of squares $F$ test was used to determine whether data were fitted best when the Hill slope, binding slope, or transducer slope ($n_B$, $n_T$, or $n_T$, respectively) were significantly different from unity, where $P < 0.05$ was considered significant.

Results

The Contribution of Slope Factors to Agonist Concentration-Response Relationships. We first evaluated the contribution of the agonist binding slope ($n_B$) or transducer slope ($n_T$) to the concentration-response curve of two agonists with different efficacies by simulating variations in $n_B$ or $n_T$ using the cooperative agonist operational model (eq. 2). We specifically wanted to evaluate a system with cooperative agonist binding; therefore, we based our simulations on CaSR activation of the CaSR. The CaSR concentration-response relationship for the CaSR’s best characterized physiologic role, inhibition of parathyroid hormone secretion, occurs over a CaSR concentration range of 0.8–1.5 mM with an EC$_{50}$ value of ~1.2 mM (Brown, 1983). Thus, for these simulations, the affinity of the agonist ($K_A$) was assumed to be 1.2 mM and $n_B$ or $n_T$ were assumed to be between 1 and 3. Simulated data were subsequently fitted to a Hill equation (eq. 9). Unsurprisingly, increasing $n_B$ or $n_T$ increases the Hill slope of the agonist concentration-response curve (Fig. 1; Table 1). Furthermore, increasing $n_T$ decreases agonist potency. Interestingly, the effect of $n_B$ on agonist potency depends on the magnitude of $n_T$ and $\tau_A$. For instance, increasing $n_B$ decreases agonist potency for higher efficacy agonists ($\tau_A$ 3). However, for lower efficacy agonists ($\tau_A$ 1), increasing $n_B$ decreases agonist potency when $n_T \leq 1$, but increases agonist potency when $n_T \geq 2$.

We next sought to directly compare the influence of the binding or transducer slope by simulating concentration-response curves for agonists with varying efficacies using the Black-Leff operational model (eq. 1, which contains transducer and binding slopes, $n_T$ and $n_B$, respectively). As can be seen in Fig. 2 and Table 2, when $n_T$ or $n_B$ are equal to 1, variations in $\tau_A$ have an identical effect on empirical agonist concentration-response parameters (potency, Hill slope, or $E_{\text{max}}$) regardless of the model. In contrast, when $n_T$ or $n_B$ are greater than 1, variations in $\tau_A$ result in major differences in the agonist concentration-response profile predicted with the two different operational models of agonism. Specifically, the Black-Leff model predicts that high-efficacy agonists have greater potency relative to affinity (due to amplification of the steps between agonist binding and response), while for low-efficacy agonists, the EC$_{50}$ value may be less than the $K_A$ value for curves that possess nonunity Hill slopes. The latter was previously noted by Black et al. (1985). Furthermore, the Hill slope decreases alongside decreases in $\tau_A$. In comparison, the
cooperative agonist operational model predicts that when $n_T = 1$, agonist EC50 may approach but not be less than its $K_A$ value, regardless of whether $n_B > 1$, and there is no effect of $\tau_A$ on the Hill slope of the agonist concentration-response curve (Fig. 2; Table 2).

**Quantification of Experimentally Derived Agonist Concentration-Response Data.** We next tested whether our simulations were recapitulated in a functional assay that measures CaSR activation. To do so, we measured Ca$^{2+}$-mediated Ca$^{2+}$ mobilization following titration of CaSR expression using a tetracycline inducible system. In the absence of tetracycline, the maximal response to Ca$^{2+}$o is approximately 50% of the maximal response obtained under full induction of receptor expression (100 ng/ml tetracycline). In this system, fitting a Hill equation (eq. 9) to both data sets indicated that the data were fitted best when the Hill slope was unchanged with different receptor expression levels, i.e., different magnitudes of $\tau_A$ ($P < 0.05$, extra sum-of-squares $F$ test; data not shown). For the CaSR, the small molecule allosteric agonists 1-(1,3-benzothiazol-2-yl)-1-(2,4-dimethyl-phenyl)ethanol (AC265347) and $R$-1-(6-methoxy-4-(trifluoromethyl)-3-biphenylyl)-N-(R)-1-phenylethyl)ethanamine (calcimimetic B) activate the CaSR with a Hill slope of 1

**TABLE 1**

Simulation of agonist concentration-response relationships upon changes in binding or transducer slopes and $\tau_A$

Data were simulated using the cooperative agonist operational model (eq. 2) and a Hill equation (eq. 9) was fitted to simulated data to determine agonist potency, maximum effect, and Hill slope.

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$\tau_A$ = 0.5

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$\tau_A$ = 3

$pEC_{50}$, agonist potency.

**Fig. 2.** Cooperative agonist binding influences agonist concentration-response relationships. Simulations demonstrating the influence of agonist efficacy ($\tau_A$) on agonist concentration-response relationships when the slope is governed by the transducer slope ($n_T$, Black-Leff operational model of agonism) or the agonist binding slope ($n_B$, cooperative agonist operational model). Data were simulated using the Black-Leff model (eq. 1) or the cooperative agonist operational model (eq. 2), where the affinity of the agonist ($K_A$) is 1.2 mM and $n_T$ and $n_B$ are between 1 and 3. Curves through the data are the fits to a Hill equation (eq. 9), where the parameters describing the fits are given in Table 2.
(Cook et al., 2015; Keller et al., 2018). Similarly, when cooperative agonist binding is prevented by removal of the CaSR’s N-terminal domain, and consequently the primary Ca\textsuperscript{2+} binding sites, the Hill slope for Ca\textsuperscript{2+} is not significantly different from unity (as shown subsequently). This provides experimental evidence that the CaSR’s transducer slope is equal to unity, and that the steep Hill slopes observed for Ca\textsuperscript{2+} at the full-length CaSR thus likely arise from a binding slope greater than 1. Thus, when fitting CaSR experimental data to the cooperative agonist operational model, n\textsubscript{T} was constrained to unity.

When the data were fitted to the classic Black-Leff model, the estimated K\textsubscript{a} value was 0.2 mM (Fig. 3A; Table 3). In comparison, the cooperative agonist operational model yielded a K\textsubscript{a} estimate of 1.1 mM, which is in close agreement with the EC\textsubscript{50} value (1.2 mM) of Ca\textsuperscript{2+} for suppressing parathyroid hormone release (Brown, 1983) and Ca\textsuperscript{2+} affinity estimates for the CaSR extracellular domain determined using spectroscopic approaches (Huang et al., 2009; Zhang et al., 2014). For both analyses, data were fitted best when the binding slope (cooperative agonist operational model) or transducer slope (Black-Leff model) were different from unity (P < 0.05, extra sum of squares F test).

To further validate our simulations in a functional assay, we next sought to quantify the affinity and efficacy of a CaSR partial agonist. To do so, we took advantage of observations that in comparison with at the WT CaSR (Fig. 3B), Ca\textsuperscript{2+} acting via the 7 transmembrane domain is a partial agonist at an N-terminally truncated CaSR (depicted in Fig. 3C) relative to the extracellular trivalent gadolinium cation (Fig. 3D). The fluorescence-activated cytometry analysis confirmed cell surface expression of the WT and N-terminally truncated CaSR (Supplemental Fig. 1). We quantified Ca\textsuperscript{2+} affinity and efficacy at the N-terminally truncated CaSR using the original Black-Leff model or the cooperative agonist operational model (Table 3). In both instances, data were best fitted when the binding slope (cooperative agonist operational model) or transducer slope (the Black-Leff model) were not different from unity (P < 0.05, extra sum of squares F test).

![Image](https://via.placeholder.com/150)

**Fig. 3.** Ca\textsuperscript{2+}-CaSR concentration-response relationships fit well to the cooperative agonist operational model. (A) Ca\textsuperscript{2+}-mediated Ca\textsuperscript{2+} mobilization at the WT CaSR following overnight receptor induction with 100 ng/ml tetracycline (tet) or in the absence of tetracycline. Data are mean ± S.D. from four independent experiments performed in duplicate. Curves through the data are the fits to the Black-Leff model (blue line) or the cooperative agonist operational model (red line), where the parameters describing the fits are given in Table 3. Although both models fit the data, the cooperative agonist operational model more accurately predicts the expected affinity of Ca\textsuperscript{2+} at the CaSR (Table 3). (B) Extracellular trivalent gadolinium (Gd\textsuperscript{3+}) and Ca\textsuperscript{2+} concentration-response curves at the WT CaSR following overnight receptor induction with 100 ng/ml tetracycline. Data are mean ± S.D. from four independent experiments performed in duplicate. Curves through the data are the fits to a four-parameter Hill equation. (C) N-terminally truncated CaSR snake diagram. (D) Gd\textsuperscript{3+} and Ca\textsuperscript{2+} concentration-response curves at an N-terminally truncated CaSR following overnight receptor induction with 100 ng/ml tetracycline. Data are mean ± S.D. from five independent experiments performed in duplicate. Curves through the data are the fits to the cooperative agonist operational model, where the parameters describing the fits are given in Table 3.

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**Table 2**

Simulation of agonist concentration-response relationships upon changes in binding or transducer slopes and $\tau_T$.

Data were simulated using the Black-Leff model (eq. 1) or the cooperative agonist operational model (eq. 2) and a Hill equation (eq. 9) was fitted to simulated data to determine agonist potency, maximum effect, and Hill slope.

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</table>

pEC\textsubscript{50}, agonist potency.
Having established that the cooperative agonist operational model accurately quantified a reduction in $Ca^{2+}$ efficacy at the N-terminally truncated receptor in comparison with at the WT receptor. This is consistent with a lower $Ca^{2+}$ $E_{\text{max}}$ value at the N-terminally truncated receptor (~60% of the maximum response stimulated by extracellular trivalent gadolinium) in comparison with WT (~100% extracellular trivalent gadolinium $E_{\text{max}}$) (Table 3). Thus, only the cooperative agonist operational model accurately estimated $Ca^{2+}$ partial agonism at the N-terminally truncated receptor.

### Quantifying Allosteric Interactions in Systems with Different Degrees of Cooperative Agonist Binding.

Having established that the cooperative agonist operational model best fitted our $Ca^{2+}$-WT CaSR concentration-response curves with Hill slopes greater than 1, we next extended this model to allow for quantification of allosteric modulation of an agonist response. The operational model of agonism and allosterism (Leach et al., 2007, 2010) (referred to herein as the original operational model of agonism and allosterism), which takes into account the allosteric effects on agonist affinity and efficacy, combines the allosteric ternary complexes model developed by Stockton et al. (1983) and Ehlert (1988) and the Black-Leff operational model of agonism. In our original model (Leach et al., 2007), the allosteric modulator can also possess intrinsic efficacy. Introduction of a slope in that model once again assumed that the slope linked occupancy to response, not to the original binding events, which were assumed to be described as simple one-to-one hyperbolic functions. Therefore, we adapted this operational model of allosterism to account for cooperative agonist binding, referred to hereinafter as the operational model of allosterism with cooperative agonist binding. To validate this operational model of allosterism with cooperative agonist binding, we reanalyzed existing data demonstrating positive and negative allosteric modulation at three model GPCRs with different agonist Hill slopes: CaSR (a class C GPCR where the primary endogenous agonist, $Ca^{2+}$, has a Hill slope of 2–4), mGlu5 (a class C GPCR where the primary endogenous agonist, L-glutamate, has a Hill slope of 1.8) (Sengmany and Gregory, 2016), and M4 mAChR (a class A GPCR where the endogenous agonist, acetylcholine, has a Hill slope of 1) (Leach et al., 2010, 2011) (Supplemental Fig. 2). In all instances, $n_T$ was assumed to be unity and all allosteric modulators were assumed to bind to a single site (i.e., the modulator binding slope is unity).

For the CaSR, we analyzed allosteric modulation of $Ca^{2+}$ by cinacalcet (PAM) or NPS2143 (NAM) (Leach et al., 2016) with the original operational model of agonism and allosterism with contaminating (i.e., ambient buffer) agonist (eqs. 5 or 6, respectively) and the newly derived operational model of allosterism with cooperative agonist binding and contaminating agonist (eqs. 7 or 8, respectively) (Fig. 4). Similar to our analysis of agonist concentration-response curves, data were fitted best when the binding slope (original operational model of agonism and allosterism) was different from unity ($P < 0.05$, extra sum of squares $F$ test). Compared with the original operational model of agonism and allosterism, the estimated affinity for $Ca^{2+}$ determined using the operational model of allosterism with cooperative agonist binding (1.4 mM) (Table 4) was once again closer to the assumed $Ca^{2+}$ affinity based on its EC$_{50}$ value for suppression of parathyroid hormone release (1.2 mM) and quantification of the $Ca^{2+}$ $K_A$ at the extracellular domain using spectroscopic approaches (3–5 mM) (Huang et al., 2009; Zhang et al., 2014). Furthermore, the estimated affinity and negative cooperativity of NPS2143 were greater (5.5- and 35-fold, respectively) when cooperative agonist binding was factored into the analysis (Table 4). For the PAM, cinacalcet,
expression of the model of allosterism with cooperative agonist binding yielded a 3-fold lower affinity estimate but an 8-fold greater magnitude of positive cooperativity.

We next analyzed allosteric modulation of glutamate at mGlu5 (eqs. 3 or 4) by a representative full NAM (2-methyl-6-(2-phenylethyl)pyridine [MPEP]) that completely inhibits glutamate-mediated activation of Ca\textsuperscript{2+} mobilization, a partial NAM (2-(2-(3-methoxyphenyl)ethenyl)-5-methylpyridine [M-5MPEP]) that only partially inhibits glutamate-mediated activation of Ca\textsuperscript{2+} mobilization (Sengmany et al., 2019), a pure PAM (N-(1,3-Diphenyl-1H-pyrazolo-5-yl)-4-nitrobenzamide [VU-29]), and a mixed PAM-agonist (1-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-(4-fluorobenzyl)oxy)ethanone [DPFE]) (Sengmany et al., 2017). Similar to analyses at the CaSR, all data were fitted best when the binding slope (operational model of allosterism with cooperative agonist binding) or transducer slope (original operational model of agonism and allosterism) was different from unity (P < 0.05, extra sum of squares F-test). However, for each modulator, the affinity and cooperativity estimates were similar (within 3-fold) irrespective of the analytical model applied (Fig. 5; Table 5). Therefore, although the glutamate-mGlu5 concentration-response relationship has a Hill slope greater than unity, quantification of allosteric interactions at mGlu5 is largely unaffected by whether the empirical slope is assumed to be determined by the transducer slope or the agonist binding slope.

For the M\textsubscript{4} mAChR, we analyzed previously published positive allosteric modulation of ACh by the PAM agonist LY20332982 in guanosine 5′-O-(3-[3H]thio)triphosphate binding assays (Leach et al., 2010) (eqs. 3 or 4). As expected, in the absence of competitive ACh binding at the M\textsubscript{4} mAChR, data for the interaction between ACh and LY20332982 were fitted best by both operational models of agonism and allosterism when the slope was not different from unity (P < 0.05, extra sum of squares F test); therefore, both equations yielded identical estimates of affinity and cooperativity (Fig. 6; Table 6).

We next sought to explain why quantification of PAM and NAM affinity and cooperativity were not greatly affected by the assignment of the slope at mGlu5, where the glutamate Hill slope is greater than unity. To do so, we simulated the interaction between an orthosteric agonist and a NAM or PAM with the operational model of allosterism with cooperative agonist binding and analyzed the simulated data with the original operational model of agonism and allosterism. For these simulations, orthosteric agonist affinity (1 μM), τ\textsubscript{A} (10), and modulator efficacy (10 nM) were held constant, and different magnitudes of positive or negative cooperativity were examined alongside changes in the magnitude of cooperative agonist binding. Consistent with our analysis of mGlu5 allosteric interaction data, when the agonist binding

### Table 4
Comparison of parameters describing CaSR allosteric interactions analyzed with different allosteric models

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ca\textsuperscript{2+} vs. Cinacalcet</th>
<th>Ca\textsuperscript{2+} vs. NPS2143</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEC\textsubscript{50}</td>
<td>eq. 5 3.3 ± 0.01</td>
<td>eq. 7 3.3 ± 0.01</td>
</tr>
<tr>
<td>pK\textsubscript{B} [K\textsubscript{A} (nM)]</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Log ( \tau_\text{A} (\tau_\text{B}) )</td>
<td>6.3 ± 0.04 (0.5)</td>
<td>5.8 ± 0.1 (1.6)</td>
</tr>
<tr>
<td>Log ( \tau_\text{B} (\tau_\text{A}) )</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Log ( \tau_\text{N} (\tau_\text{N}) )</td>
<td>n.a. (0)</td>
<td>n.a. (0)</td>
</tr>
<tr>
<td>Log ( \alpha_\beta (\alpha_\beta) )</td>
<td>0.5 ± 0.01 (3.2)</td>
<td>1.4 ± 0.1 (25)</td>
</tr>
<tr>
<td>n</td>
<td>2.8 ± 0.1 (^\dagger)</td>
<td>2.8 ± 0.1 (^\dagger)</td>
</tr>
<tr>
<td>( E_{\text{m}} ) (% ioniomycin)</td>
<td>79 ± 1.0</td>
<td>80 ± 1.0</td>
</tr>
</tbody>
</table>

\( \text{Dfd}, \text{degrees of freedom denominator}; \text{Dfn}, \text{degrees of freedom numerator}; E_{\text{m}}, \text{maximum system response}; Log \alpha_\beta (\alpha_\beta), \text{modulator cooperativity}; Log \tau_\text{A} (\tau_\text{B}), \text{Ca}\textsuperscript{2+} affinity; Log \tau_\text{B} (\tau_\text{A}), \text{modulator efficacy}; Ca\textsuperscript{2+} potency; pK\textsubscript{A}, \text{Ca}\textsuperscript{2+} affinity; pK\textsubscript{B}, \text{modulator affinity}. \)

\(^\dagger\) An F test determined that data were fitted best when the transducer or binding slopes were different from unity. The F data used to test the hypothesis that D differed from 1 were the following: eq. 5 cinacalcet P < 0.0001, P (Dfn, Dfd) 1249 (1, 2429); eq. 6 Cinacalcet P < 0.0001, P (Dfn, Dfd) 1022 (1, 908); eq. 7 cinacalcet P < 0.0001, P (Dfn, Dfd) 1241 (1, 4229); and eq. 7 NPS2143 P < 0.0001, P (Dfn, Dfd) 504.5 (1, 907).
mGlu5 allosteric interactions with glutamate in Ca\(^{2+}\) mobilization assays were analyzed with the original operational model of agonism and allosterism (eq. 3) or the operational model of allosterism with cooperative agonist binding.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glutamate vs. M-5MPEP</th>
<th>Glutamate vs. VU-29</th>
<th>Glutamate vs. MPEP</th>
<th>Glutamate vs. M-5MPEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK(_A) (M)</td>
<td>6.2 (0.6)</td>
<td>6.2 (0.6)</td>
<td>6.2 (0.6)</td>
<td>6.2 (0.6)</td>
</tr>
<tr>
<td>Log t(_A)</td>
<td>0.1 (0.01)</td>
<td>0.1 (0.004)</td>
<td>0.2 (0.0)</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td>E(_m) (% glutamate)</td>
<td>106 (10)</td>
<td>106 (10)</td>
<td>106 (10)</td>
<td>106 (10)</td>
</tr>
</tbody>
</table>

Operational Models for Receptors with Multiple Agonist Sites

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**Table 5.** Comparison of parameters describing mGlus allosteric interactions, analyzed with different allosteric models.

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**Discussion**

In the current study, we have assessed operational models of agonism and allosterism that account for receptors whose agonists bind multiple binding sites in a cooperative manner. The modified models accurately fit experimental data at an exemplar GPCR, the CaSR, which has high sensitivity for Ca\(^{2+}\) due to multiple Ca\(^{2+}\) binding sites that are linked in a positively cooperative manner.

The modified models accurately fit experimental data at an exemplar GPCR, the CaSR, which has high sensitivity for Ca\(^{2+}\) due to multiple Ca\(^{2+}\) binding sites that are linked in a positively cooperative manner. We show that agonist Hill slopes that differ from unity and remain unchanged by alterations in receptor expression levels or cellular coupling efficiencies (i.e., where \(T_A\) differs) may be indicative of cooperative agonist binding. We demonstrate that if a steep Hill slope such as that observed at the CaSR is attributed to the transducer slope rather than to the agonist binding slope, the Black-Leff operational model of agonism underestimates agonist efficacy and overestimates agonist affinity. Extension to allosteric interactions shows the importance of accounting for cooperative agonist binding, since different models fitted to the same allosteric interaction data yield divergent modifier affinity and cooperativity estimates. For instance, the original operational model of agonism and allosterism estimates lower CaSR PAM and NAM cooperativity values and higher or lower stoichiometric parameters compared to the extended Black-Leff model.
affinity values, respectively. Data simulations support these findings and demonstrate that the impact of cooperative binding on estimates of modulator affinity and cooperativity is more pronounced when the magnitude of modulator cooperativity or agonist cooperativity is increased. This was evidenced by comparing data at receptors that possess multiple agonist-receptor concentration-response relationships with Hill slopes equal to unity, it does not matter whether the slope is the margin of experimental error (\( \pm 0.2 \)) or the operational model of allosterism with cooperative agonist binding. Orthosteric agonist affinity (1 M), \( \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

\[ F_{\text{max}} (\% \text{ACh maximal}) = 112 \pm 5.0 \]

\( \delta \), degrees of freedom denominator; Dfn, degrees of freedom numerator; \( E_{\text{max}} \), maximum system response; \( \log \alpha (\beta) \), cooperativity; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

\[ F_{\text{max}} (\% \text{ACh maximum}) = 112 \pm 5.0 \]

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\[ F_{\text{max}} (\% \text{ACh maximum}) = 112 \pm 5.0 \]

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\[ F_{\text{max}} (\% \text{ACh maximum}) = 112 \pm 5.0 \]

\( \delta \), degrees of freedom denominator; Dfn, degrees of freedom numerator; \( E_{\text{max}} \), maximum system response; \( \log \alpha (\beta) \), cooperativity; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

\[ F_{\text{max}} (\% \text{ACh maximum}) = 112 \pm 5.0 \]

\( \delta \), degrees of freedom denominator; Dfn, degrees of freedom numerator; \( E_{\text{max}} \), maximum system response; \( \log \alpha (\beta) \), cooperativity; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

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\( \delta \), degrees of freedom denominator; Dfn, degrees of freedom numerator; \( E_{\text{max}} \), maximum system response; \( \log \alpha (\beta) \), cooperativity; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

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\( \delta \), degrees of freedom denominator; Dfn, degrees of freedom numerator; \( E_{\max} \), maximum system response; \( \log \alpha (\beta) \), cooperativity; \( \log \tau (\alpha) \), agonist efficacy; \( \log \tau (\alpha) \), modulator efficacy; \( n \), transducer or binding slope; \( pK_A (K_A) \), agonist affinity; \( \delta \), modulator affinity.

\[ F_{\max} (\% \text{ACh maximum}) = 112 \pm 5.0 \]
cooperativity can predict likely clinical efficacy or adverse effect liability. Inaccurate estimation of allosteric modulator affinity or cooperativity due to a failure to consider cooperative agonist binding likely also impacts interpretation of structure-function studies. If cooperativity values are narrowed, then more subtle effects of mutations on modulator cooperativity may have been missed.

In conclusion, we have validated a method for quantifying agonist and allosteric modulator actions at receptors possessing multiple agonist binding sites that interact in a cooperative manner. Our operational models of agonism and allostery with cooperative agonist binding more accurately quantify the actions of both orthosteric and allosteric drugs acting at GPCRs with cooperative agonist binding and may be used for future drug discovery efforts at these important receptors.

Appendix

Cooperative agonist operational model

A model of signal transduction by a receptor with \(n_B\) binding sites is displayed in the equilibrium:

\[
\langle n_B \rangle A + R \xrightleftharpoons{K_{A1}} A_2 R + \langle n_B - 1 \rangle A \xrightleftharpoons{K_{A2}} A_3 R + \langle n_B - 2 \rangle A \xrightleftharpoons{K_{A3}} A_{n_B} R \xrightleftharpoons{K_E} E
\]

Where \(K_E\) is the value of \(A_{n_B} R\) that elicits half the maximal system effect and agonist affinity for each site can be described by the equilibrium dissociation constants:

\[
K_{A1} = \frac{[A][R]}{[AR]}, K_{A2} = \frac{[A][AR]}{[A_2 R]}, K_{A3} = \frac{[A][A_2 R]}{[A_3 R]}, \text{etc}
\]

We see that \(K_{A1} \times K_{A2} \times K_{A3} \times \cdots \times K_{A_{n_B}} = \frac{[A]^{n_B}[R]}{[A_{n_B} R]} = K_{A_{n_B}}\), where \(K_A\) is the geometric mean of the individual equilibrium dissociation constants.

For simplicity, the receptor is considered either empty (R) or fully occupied (\(A_{n_B} R\)): \(\langle n_B \rangle A + R \xrightleftharpoons{K_{A1}} A_{n_B} R \xrightleftharpoons{K_E} E\)

\[
K_{A_{n_B}} = \frac{[A]^{n_B}[R]}{[A_{n_B} R]}
\]

The total receptor concentration can be expressed as:

\[
[R_0] = [R] + [A_{n_B} R]
\]

where

\[
[R] = \frac{K_{A_{n_B}}[A_{n_B} R]}{[A]^{n_B}}
\]

therefore

\[
[R_0] = \frac{K_{A_{n_B}}[A_{n_B} R]}{[A]^{n_B}} + [A_{n_B} R]
\]

\[
[R_0] = [A_{n_B} R] \left( \frac{K_{A_{n_B}}}{[A]^{n_B}} + 1 \right)
\]

\[
[R_0] = [A_{n_B} R] \left( \frac{K_{A_{n_B}} + [A]^{n_B}}{[A]^{n_B}} \right)
\]

Receptor occupancy is thus denoted:

\[
[A_{n_B} R] = \frac{[R_0][A]^{n_B}}{K_{A_{n_B}} + [A]^{n_B}}
\]

In accordance with the scheme of the operational model of agonism, the logistic function for the transduction of occupancy into response is:

\[
E = \frac{E_m A_{n_B} R R^{\tau} [R_0]^{n_B} [A]^{n_B}}{K_{E} R^{\tau} + [A]^{n_B} [R_0]^{n_B} [A]^{n_B}}
\]

Multiplying numerator and denominator by \((K_A^{n_B} + [A]^{n_B})^{n_T}\) gives:

\[
E = \frac{E_m [R_0]^{n_T} [A]^{n_B} R^{\tau} K_{E}^{n_T} (K_A^{n_B} + [A]^{n_B})^{n_T}}{[R_0]^{n_T} [A]^{n_B} R^{\tau} (K_A^{n_B} + [A]^{n_B})^{n_T} + [R_0]^{n_T} [A]^{n_B} R^{\tau} (K_A^{n_B} + [A]^{n_B})^{n_T}}
\]

Dividing through by \(K_E\) and redefining \(\frac{[R_0]}{K_E}\) as \(\tau_A\) gives an operational model of agonism for a receptor with \(n_B\) binding sites (Eq 2 in the main text):

\[
E = \frac{E_m \tau_A [A]^{n_B} R^{\tau}}{(K_A^{n_B} + [A]^{n_B})^{n_T} + \tau_A [A]^{n_B} R^{\tau}}
\]

For use in Graphpad Prism or similar software, the above equation is described by the following notations, where \(n_T\) or \(n_B\) will likely need to be fixed to a known or theoretical value to fit real experimental data to this equation, and where a “Basal” response parameter is introduced to accommodate ligand-independent effects that deviate from zero:

\[
KA = 10^{\tau}\log KA
\]

\[
A = 10^X
\]

\[
tau = 10^\tau\log tau
\]

\[
Part1 = (Em - Basal)^{\tau_1}(A^{n_B})
\]

\[
Part2 = \tau_A (A^{n_B}) + (KA^{n_B})
\]

\[
Y = Basal + (Part1^{n_T})/(Part2^{n_T})
\]

Operational model of allostery with cooperative agonist binding

In a ternary complex consisting of a receptor (lacking constitutive activity), an orthosteric agonist that binds \(n_B\) binding sites, and an allosteric modulator, the stimulus-generating species are \(A_{n_B} R\), \(BR\) and \(A_{n_B} RB\). A model of ligand-receptor interactions in this ternary complex is displayed in the equilibrium:

\[
\langle n_B \rangle A + R \xrightleftharpoons{K_{A1}} A_{n_B} R; B + R \xrightleftharpoons{K_E} BR\text{ and}
\]

\[
\frac{K_B}{A_{n_B} R + B} \xrightleftharpoons{K_{A2}} A_{n_B} RB
\]

Where \(K_{A_{n_B}} = \frac{[A]^{n_B}[R]}{[A_{n_B} R]}, K_B = \frac{[B][R]}{[BR]}, \text{ and } K_{E} = \frac{[A]^{n_B}[R]}{[A_{n_B} RB]}.\)
From the latter expression we obtain:

$$\alpha = \frac{K_B[A_{nB}RB]}{A_{nB}[B]}$$

Assuming the pharmacological effect ($E$) is a function of the total stimulus ($S_T$) arising from the sum of the stimuli generated from each individual ligand-bound receptor species, then:

$$S_T = S_{A_{nB}R} + S_{RB} + S_{A_{nB}RB}$$

It is further assumed that the stimulus ($S$) generated from each individual ligand-bound receptor species is proportional to the product of the intrinsic efficacy of the ligand ($e$) and the concentration of the ligand-bound receptor species, thus:

$$S_{A_{nB}R} = e_{A_{nB}}[A_{nB}R]; \ S_{RB} = e_B[BR]; \ S_{A_{nB}RB} = e_{A_{nB}B}[A_{nB}RB];$$

and

$$e_{A_{nB}}$$ denotes the intrinsic efficacy of the orthosteric agonist, $e_B$ denotes the intrinsic efficacy of the allosteric modulator, and $\beta$ is a coupling factor that describes the effect of the allosteric modulator on the intrinsic efficacy of the orthosteric agonist when the two ligands are bound to the same receptor.

Thus, the effect ($E$) of an agonist in the presence of an allosteric modulator is processed through the following logistic equation

$$E = \frac{E_m S_T^{nT}}{K_S^{nT} + S_T^{nT}}$$

where $E_m$ denotes the maximum possible response, $K_S$ denotes a constant that governs the efficiency of stimulus-response coupling and $nT$ denotes a logistic slope factor (the transducer slope).

If we consider the total receptor concentration:

$$[R_0] = [R] + [A_{nB}R] + [BR] + [A_{nB}RB]$$

where

$$[A_{nB}R] = \frac{[R_0][A]^{nB}}{[A]^{nB} \left( 1 + \frac{KB}{KC_{138}} \right) + K_A^{nB} \left( 1 + \frac{KB}{KC_{138}} \right)}$$

$$[BR] = \frac{[R_0][B]}{[B] \left( 1 + \frac{KB}{KC_{138}} \right) + K_B \left( 1 + \frac{KB}{KC_{138}} \right)}$$

and

$$[A_{nB}RB] = \frac{[R_0][A]^{nB}}{[A]^{nB} \left( 1 + \frac{KB}{KC_{138}} \right) + K_A^{nB} \left( 1 + \frac{KB}{KC_{138}} \right)}$$

substituting the above terms into $E = \frac{E_m S_T^{nT}}{K_S^{nT} + S_T^{nT}}$ gives the following operational model of allosterism at a receptor with $nB$ orthosteric agonist binding sites (Eq 4 in the main text):

$$E = \frac{E_m \left( \tau_A[A]^{nB}(K_B + \alpha[B]) + \tau_B[B][K_A^{nB}] \right)^{nT}}{\left( [A]^{nB}K_B + K_A^{nB}K_B + K_A^{nB}[B] + \alpha[A]^{nB}[B] \right)^{nT}}$$

where

$$\tau_A = \frac{e_{A_{nB}}[R_0]}{K_S}$$

and

$$\tau_B = \frac{e_B[R_0]}{K_S}$$

For use in Graphpad Prism or similar software, the above equation is described by the following notations, where $nT$ or $nB$ will likely need to be fixed to a known or theoretical value to fit real experimental data to this equation:

$$KA = 10^\logKA$$

$$KA = 10^\logKB$$

$$tauA = 10^\logtauA$$

$$tauB = 10^\logtauB$$

$$A = 10^\logA$$

$$alpha = 10^\logalpha$$

$$beta = 10^\logbeta$$

$$B = 10^LogAllo$$

Part1 = $\log( (Em - Basal) / (Stim - nT) ) + (1/nT)$

Part2 = $\log( (KA^{nB}KB + (KA^{nB}KB + B^{(KA^{nB}B + alpha^{(A^{nB}B)}B)})$ Stimm = Part1/Part2

For the purposes of the current study, the model has been simplified further to enable analysis of data when the orthosteric agonist is a full agonist. Therefore, equation 4 in the manuscript reduces to:

$$E = \frac{E_m \left( \tau_A[A]^{nB}(K_B + \alpha[B]) + \tau_B[B][K_A^{nB}] \right)^{nT}}{K_A^{nB}[K_B + [B]]^{nT} + \left( \tau_A[A]^{nB}(K_B + \alpha[B]) + \tau_B[B][K_A^{nB}] \right)^{nT}}$$

Dividing through by $\tau_A^{nT}$, and defining $[EC_{50}] = K_A/\tau_A$ yields the following expression:

$$E = \frac{E_m \left( [A]^{nB}(K_B + \alpha[B]) + \tau_B[B][EC_{50}]^{nB} \right)^{nT}}{[EC_{50}]^{nB}[K_B + [B]]^{nT} + \left( [A]^{nB}(K_B + \alpha[B]) + \tau_B[B][EC_{50}]^{nB} \right)^{nT}}$$

Authorship Contributions

Participated in research design: Gregory, Christopoulos, Leach.

Conducted experiments: Diao.

Contributed new reagents or analytic tools: Giraldo, Christopoulos.

Performed data analysis: Diao, Gregory, Giraldo, Leach.
Wrote or contributed to the writing of the manuscript: Gregory, Giraldo, Christopoulos, Leach.

References

Address correspondence to: Katie Leach, Drug Discovery Biology and Department of Pharmacology, Monash Institute of Pharmaceutical Sciences, Monash University, 399 Royal Parade, Parkville, Melbourne, VIC 3052, Australia. E-mail: katie.leach@monash.edu