Critical Re-evaluation of the Slope Factor of the Operational Model of Agonism Short Title: Slope Factor of the Operational Model of Agonism

Jan Jakubík (✉ jan.jakubik@fgu.cas.cz)

Czech Academy of Sciences

Alena Randáková

Czech Academy of Sciences

Dominik Nelic

Czech Academy of Sciences

Research Article

Keywords: Receptor theory, operational model of agonism, operational efficacy, slope factor

Posted Date: September 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-900678/v1>

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Critical re-evaluation of the slope factor of the operational model of agonism

Short title: Slope factor of the operational model of agonism

Jan Jakubík, Alena Randáková, Dominik Nelic

Institute of Physiology Czech Academy of Sciences, Prague, Czech Republic

Correspondence: Jan Jakubik, Inst. of Physiology CAS, Vídeňská 1083, 142 20 Praha, Czech Republic, jan.jakubik@fgu.cas.cz

Abstract

Although being a relative term, agonist efficacy is a cornerstone in the proper assessment of agonist selectivity and signalling bias. The operational model of agonism (OMA) has become successful in the determination of agonist efficacies and ranking them. In 1985, Black et al. introduced the slope factor to the OMA to make it more flexible and allow for fitting steep as well as flat concentration-response curves. Functional analysis of OMA demonstrates that the slope factor implemented by Black et al. affects relationships among parameters of the OMA. Fitting of the OMA with Black et al. slope factor to concentration-response curves of experimental as well as theoretical data (homotropic allosteric modulation, substrate inhibition and non-competitive auto-inhibition) resulted in wrong estimates of operational efficacy and affinity. In contrast, fitting of the OMA modified by the Hill coefficient to the same data resulted in correct estimates of operational efficacy and affinity. Therefore OMA modified by the Hill coefficient should be preferred over Black et al. equation for ranking of agonism and subsequent analysis, like quantification of signalling bias, when concentration response curves differ in the slope factor.

Keywords

Receptor theory; operational model of agonism; operational efficacy; slope factor

Introduction

Since its introduction in 1983, the operational model of agonism (OMA) has become a golden standard in an evaluation of agonism and subsequently also of signalling bias¹. The OMA describes the response of the system as a function of ligand concentration using three parameters: 1) The equilibrium dissociation constant of agonist (K_A) to the receptor initiating functional response; 2) The maximal possible response of the system (E_{MAX}); 3) The operational efficacy of agonist (τ). Original OMA describes the dependence of functional response on the concentration of ligand by rectangular hyperbola (Eq. 2). In practice, positive cooperativity or positive feedback lead to steep and negative cooperativity or negative feedback lead to flat concentration-response curves that the OMA does not fit to. Therefore, an equation intended for the description of non-hyperbolic concentration curves (Eq. 7), particularly for systems where the number of receptors varies, was introduced by Black et al.². Since then, Eq. 7 is commonly used. The presented mathematical analysis of Eq. 7 shows that the slope factor affects the relationship between observed maximal response to agonist and operational efficacy and the relationship between the concentration of agonist for half-maximal response and its equilibrium dissociation constant. The presented case study shows that fitting Eq. 7 results in wrong estimates of model parameters.

The presented case study shows that the use of the Hill coefficient as a slope factor to the original rectangular hyperbola of OMA instead of the slope factor by Black et al. is a viable alternative. The Hill–Langmuir equation was originally formulated to describe the cooperative binding of oxygen molecules to haemoglobin³. The Hill coefficient reflects the sense and magnitude of cooperativity among concurrently binding ligands. A value of the Hill coefficient lower than 1 indicates negative cooperativity that is manifested as a flat concentration-response curve. The smaller value of the Hill coefficient the flatter curve is. A value of the Hill coefficient greater than 1 indicates positive cooperativity that is manifested as a steep concentration-response curve. The greater value of the Hill coefficient the steeper curve is. In contrast to Black et al. slope factor, the proposed implementation of the Hill coefficient (Eq. 10) does not affect the centre or asymptotes of the hyperbola describing concentration-response curves. Therefore, the Hill coefficient does not affect relationships among three parameters of the OMA, K_A , E_{MAX} and τ . This makes OMA (Eq. 11) more practical in many ways.

The general concept of the operational model of agonism

In general, OMA consist of two functions. One function describes the binding of an agonist to a receptor as the dependence of the concentration of agonist-receptor complexes [AR] on the

concentration of an agonist [A]. The second function describes the dependence of functional response (E) to an agonist on [AR]. OMA expresses the dependence of E on [A].

Rectangular hyperbolic OMA

Definition of OMA

In the simplest case, when both binding and response function are described by rectangular hyperbola (Supplementary Information Figure S1), the resulting function is also rectangular hyperbola. For example, in bi-molecular reaction, the dependence of ligand binding to the receptor [AR] is described by Eq. 1 where [A] is the concentration of ligand and K_A is its equilibrium dissociation constant that represents the concentration of ligand at which half of the total number of receptors, R_T , is bound to the ligand and the other half of the receptors is free.

$$[AR] = \frac{R_T[A]}{[A] + K_A} \quad \text{Eq. 1}$$

If the bound ligand is an agonist, it activates the receptor and produces functional response E. Response as a function of agonist binding (agonist-receptor complexes [AR]) is given by Eq. 2.

$$E = \frac{E_{MAX}[AR]}{[AR] + K_E} \quad \text{Eq. 2}$$

Where E_{MAX} is the maximum possible response of the system and K_E is the value of [AR] that elicits a half-maximal effect. Various agonists produce a functional response of different strengths. The OMA was postulated to introduce the “transducer ratio” τ that is given by Eq. 3.

$$\tau = \frac{R_T}{K_E} \quad \text{Eq. 3}$$

The substitution of Eq. 2 with Eq. 1 and Eq. 3 gives Eq. 4.

$$E = \frac{[A]\tau E_{MAX}}{[A](\tau+1) + K_A} \quad \text{Eq. 4}$$

Analysis of OMA

Eq.4 is the equation of OMA¹. It has three parameters: The equilibrium dissociation constant of agonist (K_A) at the response-inducing state of the receptor that is specific to a combination of ligand and receptor. The maximal possible response of the system (E_{MAX}) is specific to the system. And the “transducer ratio” (τ) that is specific to a combination of ligand and system. Eq. 4 is a rectangular hyperbola with the horizontal asymptote, the observed maximal response to agonist A (E'_{MAX}), given by Eq. 5.

$$E'_{MAX} = \frac{\tau}{\tau+1} E_{MAX} \quad \text{Eq. 5}$$

A more efficacious agonist (having a high value of parameter τ) elicits higher E'_{MAX} than less efficacious agonists (having a low value of parameter τ). Thus, τ is actually operational efficacy. The relationship between parameter τ and E'_{MAX} is hyperbolic meaning that two highly efficacious agonists (e.g., τ values 10 and 20) differ in E'_{MAX} values less than two weak agonists (e.g., τ values 0.1 and 0.2).

In Eq. 4, the concentration of agonist A for half-maximal response (EC_{50}), is given by Eq. 6.

$$EC_{50} = \frac{K_A}{\tau+1} \quad \text{Eq. 6}$$

According to Eq. 6, for $\tau > 0$, the EC_{50} value is always lower than the K_A value. The K_A to EC_{50} ratio is greater for efficacious agonists than for weak agonists. Similarly to E'_{MAX} , the relationship between parameter τ and EC_{50} is hyperbolic. In contrast to E'_{MAX} values, the ratio K_A to EC_{50} ratio is more profound for two highly efficacious agonists (e.g., τ values 10 and 20) than two weak agonists (e.g., τ values 0.1 and 0.2).

Limitations of OMA

The OMA has several weak points. The major drawback of OMA is the lack of physical basis of the agonist equilibrium dissociation constant K_A . In the Eq. 2 considers agonist binding [AR] refers to agonist binding to the conformation from which the functional response is initiated. The agonist binding to the receptor in an inactive conformation is not observed in the response ($K_E \rightarrow \infty$; $\tau = 0$). In the radioligand binding experiments agonists bind to all receptor conformations including the inactive ones. For various reasons the receptors in the conformation activating functional response may be scarce or absent from radioligand binding experiments. Then it may be impossible to determine the K_A value in the radioligand binding experiments. All three parameters of OMA (E_{MAX} , K_A and τ) are interdependent⁴. To fit Eq. 4 to the experimental data one of the parameters must be fixed. Thus, the maximal response of the system E_{MAX} has to be determined before fitting Eq. 4. It can be achieved by comparing functional response to a given agonist at system with reduced population of receptors by irreversible alkylation⁵. Another limitation of the OMA is that the shape of the functional response is a rectangular hyperbola.

Non-hyperbolic OMA

Definition of non-hyperbolic OMA

In practice, concentration-response curves steeper or flatter than the ones described by Eq. 4 are observed. In such cases, Eq. 4 does not fit experimental data. As stated by the authors, Eq. 7 was devised for non-hyperbolic dependence of functional response on concentration of agonist².

$$E = \frac{[A]^n \tau^n E_{MAX}}{[A]^n \tau^n + ([A] + K_A)^n} \quad \text{Eq. 7}$$

Analysis of non-hyperbolic OMA

Introduced power factor **n** changes slope and shape of functional response curve (Supplementary information Figure S3). Nevertheless, Eq. 7 as a mathematical function has rectangular asymptotes: The horizontal asymptote ($[A] \rightarrow \pm\infty$) E'_{MAX} is given by Eq. 8 and vertical asymptote ($E \rightarrow \pm\infty$) is equal to $-K_A/(\tau+1)$ (Supplementary Information Eq. A2 and Eq. A3). From Eq. 7, the EC_{50} value is given by Eq. 9.

$$E'_{MAX} = \frac{\tau^n E_{MAX}}{1 + \tau^n} \quad \text{Eq. 8}$$

$$EC_{50} = \frac{K_A}{(2 + \tau^n)^{\frac{1}{n}} - 1} \quad \text{Eq. 9}$$

Evidently, the introduced slope factor **n** affects both the observed maximal response E'_{MAX} and half-efficient concentration of agonist EC_{50} (Figure 1A and C). The influence of the slope factor on E'_{MAX} is **bidirectional** (Supplementary Information Table S1, Figure S2). For operational efficacies $\tau > 1$, an increase in the value of slope factor increases E'_{MAX} . (Figure 1A and 2A blue lines). For operational efficacies $\tau < 1$, an increase in slope factor decreases E'_{MAX} (Figure 1C and 2A yellow lines). The effect of slope factor on E'_{MAX} is the most eminent for low values of operational efficacy τ , making the estimation of model parameters of weak partial agonists impractical. Imagine full agonist $\tau=10$ and very weak agonist $\tau=0.1$. For $n=1$: Full agonist E'_{MAX} is 90 % and weak agonist E'_{MAX} is 10 % of system E_{MAX} . For $n=2$: Full agonist E'_{MAX} is 99 % (one-tenth more) and weak agonist E'_{MAX} is just 1 % (ten times less).

Figure 1 Theoretical concentration curves

Theoretical response curves according to Eq. 7, left and Eq. 11, right. Simulation parameters: $E_{MAX} = 1$; $\tau = 3$ (top) or $\tau = 0.3$ (bottom); $K_A = 10^{-6}$. Values of slope factors are listed in the legend.

An increase in the value of the slope factor increases the EC_{50} value (Figure 2B). Again, the effect of the slope factor on the EC_{50} value is more eminent at low values of operational efficacy τ (red lines). Paradoxically, any combination of operational efficacy τ and slope factor fulfilling the inequality in Figure 2C (blue area) results in EC_{50} values **greater** than K_A (e.g., Figure 1C, yellow lines). For example, $EC_{50} > K_A$ applies if $\tau = 0.5$ and $n > 1.6$, or if $\tau = 1$ and $n > 1.6$, or if $\tau = 1.5$ and $n > 2.15$. The upper asymptote of inequality is 2. Thus, the possibility of $EC_{50} > K_A$ applies to $\tau < 2$.

Figure 2 Analysis of Black & Leff equation (Eq. 7)

A, Dependency of observed E'_{MAX} to system E_{MAX} ratio (ordinate) on slope factor n (abscissa) and operational efficacy τ (legend). B, Dependency of EC_{50} to K_A ratio (ordinate) on slope factor n (abscissa) and operational efficacy τ (legend). C, Inequality plot of slope factor n (abscissa) and operational efficacy τ (ordinate) yielding half-efficient concentration EC_{50} greater than equilibrium dissociation constant K_A .

The operational efficacy τ may be also considered as a measure of “receptor reserve”. In the system with a relatively small capacity of a functional response output, the full agonist reaches its maximal response before reaching full receptor occupancy. Thus, the agonist EC_{50} value is lower than its affinity for the receptor. The smaller occupancy fraction is needed for the full response to a given agonist the greater is difference between agonist EC_{50} and K_A values. According to OMA (Eq. 2), the relation between EC_{50} and K_A is described by Eq. 6. The greater value of operational efficacy τ , the smaller EC_{50} value and the greater the difference from K_A . Thus, the value of operational efficacy τ is a measure of receptor reserve of a given agonist at a given system. In a system with a large capacity of functional output, agonists do not have a receptor reserve and must reach full receptor occupancy to elicit a full signal. In such a system,

even full agonists have small operational efficacies. Thus, the parameter τ is specific to a combination of ligand and system.

Nevertheless, for agonists that elicit at least some response in a given system, the parameter τ must be greater than 0. Then according to Eq.6 of the operational model of agonism, the EC_{50} value must be smaller than the K_A value. In principle, the EC_{50} value greater than the K_A can be achieved only by some parallel mechanism that increases the apparent K'_A , provided that a difference K'_A to K_A is greater than EC_{50} to K_A . For example, such mechanism may be negative allosteric modulation of agonist binding or non-competitive inhibition of functional response.

Limitations of the non-hyperbolic OMA

Besides all limitations of the hyperbolic OMA, the non-hyperbolic version of OMA has additional drawbacks. The most important is the lack of mechanistic background for factor n . Exponentiation of agonist concentration $[A]$ to power factor n results in S-shaped curves. As it will be shown later, a combination of common binding mechanisms and functional responses does not result in S-shaped curves. Importantly, as shown above, exponentiation of operational efficacy τ to power factor n breaks the logical relationship between observed maximal response E'_{MAX} and operational efficacy τ . That, as it will be shown later, impedes correct estimation of τ and K_A values.

OMA with Hill coefficient

Definition of OMA with Hill coefficient

Hill coefficient may serve as an alternative slope factor in the OMA. Hill equation incorporates the Hill coefficient as a slope factor to rectangular hyperbola³. The major advantage of the Hill coefficient as a slope factor is that it allows for a change in the eccentricity (vertices) of the hyperbola-like curves without changing centre (EC_{50}) and asymptotes (E'_{MAX}) (Supplementary Information Figure S3). The Hill–Langmuir equation published in 1910 can be formulated as Eq. 10.

$$E = \frac{[A]^{nH} E'_{MAX}}{[A]^{nH} + EC_{50}^{nH}} \quad \text{Eq. 10}$$

Where nH is the Hill coefficient. Substitution of E'_{MAX} by Eq. 5 and EC_{50} by Eq. 6 gives Eq. 11.

$$E = \frac{[A]^{nH} \frac{\tau}{\tau+1} E_{MAX}}{[A]^{nH} + \left(\frac{K_A}{\tau+1}\right)^{nH}} \quad \text{Eq. 11}$$

As expected, the Hill coefficient does not influence maximal observed response E'_{MAX} either half-efficient concentration of agonist EC_{50} (Figure 1B and D).

Implications of OMA with Hill coefficient

Analysis of the OMA with slope factor by Black et al. (Eq. 7) have shown that the slope factor n has a bidirectional effect on the relationship between the parameters E'_{MAX} and τ . and that the slope factor n affects the relationship between the parameters EC_{50} and K_A . In contrast, in Eq. 11 neither value of E'_{MAX} nor the value of EC_{50} is affected by the Hill coefficient (Figure 1B and D). The parameters E'_{MAX} and EC'_{50} can be readily obtained by fitting Eq. 10 to the single concentration-response data.

Limitations of OMA with Hill coefficient

The major criticism of the Hill equation is its parsimonious character. It is relatively simple and its parameters are easy to estimate. However, as a model, it is just an approximation. In an experiment, the slope of the concentration-response curve different from unity may be a result of the parallel signalling mechanism providing feedback or allosteric cooperativity. E.g., positive cooperativity results in steep concentration-response curves and negative cooperativity results in flat concentration-response curves.

The case study

How fitting the Black & Leff equation to experimental data can affect estimates of the operational efficacy and subsequent analysis is demonstrated on the following example of measurement of the GTP γ S binding as a functional response of M_2 receptor to muscarinic agonists carbachol and oxotremorine (Figure 3). At G_{i1} G-protein (Figure 3, left) carbachol stimulated the GTP γ S binding more than oxotremorine. In contrast, at G_{oA} G-protein oxotremorine stimulated GTP γ S binding slightly more than carbachol (Figure 3, right). While the functional response to carbachol has normal slope ($n_H \approx 1$), the response to oxotremorine is shallow ($n_H = 0.84$ and 0.64 , respectively) (Table 1). Fitting the Black & Leff equation (Eq. 10) to the experimental data gives the overlapping curves for carbachol and very similar curves for oxotremorine (Figure 3). Contra intuitively, while at G_{oA} the maximal observed response E'_{MAX} to oxotremorine is higher than E'_{MAX} of carbachol (0.44 vs. 0.42), the estimate of the operational efficacy τ of oxotremorine is lower than τ of carbachol (0.55 vs. 0.71). Subsequently, relative intrinsic activity (RA_i)⁶ based on EC_{50} and E'_{MAX} values for oxotremorine is higher at G_{oA} than G_{i1} (21 vs. 18), suggesting slight oxotremorine bias towards G_{oA} . But bias factor⁷ based on τ and K_A values suggest (3.6-fold) bias of oxotremorine towards G_{i1} .

Figure 3 Functional response of muscarinic M₂ receptors

GTP γ S binding to G_{i1} (left) or G_{oA} (right) G-proteins upon stimulation of M₂ muscarinic receptors by carbachol (red) or oxotremorine (blue) at the concentration indicated on the x-axis is expressed as the fraction of maximal response of the system (E_{MAX}). Curves are from a representative experiment performed in quadruplicates. Solid curves, Hill equation (Eq. 10). Dotted curves, Black & Leff equation (Eq. 7). Parameter estimates are in Table 1.

Table 1 Results of fitting Black & Leff and Hill equation to experimental data in Figure 3.

Black&Leff (Eq. 7) and Hill equation (Eq. 10) were fitted to concentration-response data of GTP γ S binding to G-proteins upon stimulation of M₂ receptors by carbachol or oxotremorine in Figure 3. The E_{MAX} was fixed to 1.

	Hill	logEC ₅₀	E' _{MAX}	n _H
G _{i1}	carbachol	-5.11 ± 0.06	0.4756 ± 0.0174	0.99 ± 0.10
	oxotremorine	-6.43 ± 0.04	0.4167 ± 0.0090	0.84 ± 0.05
G _{oA}	carbachol	-6.09 ± 0.03	0.4204 ± 0.0068	0.96 ± 0.06
	oxotremorine	-7.39 ± 0.04	0.4433 ± 0.0067	0.64 ± 0.04
	Black & Leff	logK _A	τ	n
G _{i1}	carbachol	-4.70 ± 0.17	0.906 ± 0.048	0.96 ± 0.16
	oxotremorine	-5.98 ± 0.10	0.610 ± 0.027	0.73 ± 0.07
G _{oA}	carbachol	-5.81 ± 0.10	0.705 ± 0.026	0.94 ± 0.10
	oxotremorine	-6.47 ± 0.13	0.546 ± 0.036	0.45 ± 0.05

OMA of homotropic allosteric modulation

OMA of homotropic allosteric of binding

The simplest mode of interaction that leads to variation in the slope of concentration-response curves is allosteric modulation. Let's take for example homotropic allosteric modulation and suppose that ligand A binds to the orthosteric (AR) and the allosteric binding site (RA) with the same affinity. For the sake of simplicity suppose that a ligand A activates the receptor only from the orthosteric binding site. And that A bound to the orthosteric binding site (AR) has the same operational efficacy as A in the ternary complex (ARA) (Figure 4). In such a case, the amount of active complexes AR and ARA is given by Eq. 12.

$$[AR] + [ARA] = \frac{R_T[A]}{[A] + K_A \frac{[A] + K_A}{\alpha[A] + K_A}} \quad \text{Eq. 12}$$

Where α is the factor of binding cooperativity. Values of α greater than 1 denote positive cooperativity. Values of α smaller than 1 denote negative cooperativity. The substitution of Eq. 12 and Eq. 3 into Eq. 2 gives Eq. 13 (Supplementary Information Eq. A18).

$$E = \frac{E_{MAX}\tau[A](K_A + \alpha[A])}{K_A^2 + 2[A]K_A + \alpha[A]^2 + \tau[A](K_A + \alpha[A])} \quad \text{Eq. 13}$$

Allosteric modulation does not affect observed maximal response E'_{MAX} (Supplementary Information Eq. A23). For equipotent homotropic allosteric modulation, the value of apparent K_A (K'_A) is given by Eq. 14 (Supplementary information Eq. A29) and the value of half-efficient concentration is given by Eq. 15.

$$K'_A = \frac{K_A}{\sqrt{\alpha}} \quad \text{Eq. 14}$$

$$EC_{50} = \frac{K'_A}{\tau + 1} \quad \text{Eq. 15}$$

As can be seen in Figure 4, positive cooperativity results in a steep curve and a decrease in the apparent value of K_A . On the other hand, negative cooperativity results in a flat curve and an increase in the apparent value of K_A .

Figure 4 Homotropic allosteric modulation of binding

Dots, functional response to an agonist ($\tau=0.3$, $E_{MAX}=1$, $R_T=1$) acting at two sites with the same $K_A = 10^{-6}$ M exerting binding cooperativity α indicated in the legend, according to Eq. 13. Full lines, left, Black & Leff equation (Eq. 7), right, Hill equation (Eq. 10) fitted to the data. Parameter estimates are in Table 2.

As can be seen in Figure 3 by the naked eye, the Hill equation fits the data better than the Black & Leff equation. Importantly, fitting the Hill equation gives correct estimates of apparent maximal response E'_{MAX} , while fitting the Black & Leff equation results in overestimated E'_{MAX} values in the case of negative cooperativity ($\alpha < 1$) and slightly underestimated E'_{MAX} values in the case of positive cooperativity (Table 2). As result, fitting the Hill equation gives correct estimates of operational efficacies τ . It gives also correct estimates of half-efficient concentrations EC_{50} and thus correct values of apparent as well as real equilibrium dissociation constant K_A . In contrast, fitting the Black & Leff equation gives correct parameter estimates only in the case of neutral cooperativity ($\alpha=1$). In the case of negative cooperativity ($\alpha < 1$) values of τ and pK_A are underestimated. In the case of positive cooperativity ($\alpha > 1$) values of τ and pK_A are overestimated.

Table 2 Results of fitting Black & Leff and Hill equations to the model of homotropic allosteric modulation of binding

Balck & Leff (Eq. 7) and Hill (Eq. 10) were fitted to model data (Eq. 13) with E_{MAX} fixed to 1. Apparent pK'_A values were calculated from model Eq. 13 according to Eq. 14 or from EC_{50} values according to Eq. 11.

Model Eq. 13 and 14				Black & Leff			Hill				
T	pKA	α	pK'_A	n	τ	pKA	nH	EC50	E'MAX	τ	pK'_A
0.3	6.00	0.2	5.65	0.59	0.123	5.30	0.72	5.80	0.23	0.3	5.65
0.3	6.00	0.5	5.85	0.78	0.210	5.69	0.87	5.98	0.23	0.3	5.85
0.3	6.00	1.0	6.00	1.00	0.300	6.00	1.00	6.11	0.23	0.3	6.00
0.3	6.00	2.0	6.15	1.35	0.411	6.32	1.14	6.25	0.23	0.3	6.15
0.3	6.00	5.0	6.35	1.54	0.461	6.58	1.33	6.44	0.23	0.3	6.35

Fitting Eq. 13 with fixed system E_{MAX} to the model of functional response homotropic allosteric modulation of binding yields correct parameter estimates that are associated with the low level of uncertainty (Supplementary Information Figure S6).

OMA of homotropic allosteric modulation of efficacy

Homotropic allosteric modulation of agonist binding may result in the change in operational efficacy of agonist τ (Figure 5). If the operational efficacy of AR is τ and the operational efficacy of ARA is $\beta\tau$ (K_E/β) then the apparent activation constant K'_E changes with the concentration of an agonist according to Eq. 16.

$$K'_E = K_E \frac{[A] + K_A}{\beta[A] + K_A} \quad \text{Eq. 16}$$

Where β is the cooperativity factor of efficacy. Values of β greater than 1 denote positive cooperativity, a decrease in apparent K'_E . Values of β smaller than 1 denote negative cooperativity, an increase in apparent K'_E .

For the sake of simplicity suppose neutral cooperativity ($\alpha = 1$) in the binding of A to the orthosteric and allosteric sites. Then agonist binding described by Eq. 12 simplifies to Eq. Substitution of K_E by Eq. 16 and ($[AR] + [ARA]$) by Eq. 1 in Eq. 2 gives Eq. 17 (Supplementary Information Eq. A34).

$$E = \frac{[A]\tau E_{MAX}(\beta[A] + K_A)}{K_A^2 + 2[A]K_A + [A]^2 + \tau[A](\beta[A] + K_A)} \quad \text{Eq. 17}$$

The apparent maximal response E'_{MAX} is given by Eq. 18 (Supplementary Information Eq. A39).

$$E'_{MAX} = E_{MAX} \frac{\beta\tau}{1+\beta\tau} \quad \text{Eq. 18}$$

Thus, apparent operational efficacy τ' is given by Eq. 19.

$$\tau' = \beta\tau \quad \text{Eq. 19}$$

The EC_{50} value in homotropic allosteric modulation of efficacy is given by Eq. 20 (Supplementary information Eq. A42).

$$EC_{50} = \pm \frac{\sqrt{K_A^2(4\beta^2\tau + \beta^2(\tau^2 - 4\tau + 8) + 4\beta(\tau - 2) + 4) \pm K_A(\beta(\tau - 2) + 2)}}{2\beta(1 + \beta\tau)} \quad \text{Eq. 20}$$

Both positive ($\beta > 1$) and negative ($\beta < 1$) cooperativity in operational efficacy lead to steep concentration-response curves (Figure 5, Table 3). Obviously, the Hill equation fits the data better than the Black & Leff equation. Strong negative cooperativity ($\beta = 0.2$) results in bell-shaped concentration-response curve (Figure 5, dark blue). Except for the bell-shaped curve ($\beta=0.2$), fitting of the Hill equation gives correct estimates of apparent maximal response E'_{MAX} and thus correct estimates of apparent operational efficacy τ' . In contrast, fitting the Black & Leff equation results in overestimated τ values. In the case of positive cooperativity ($\beta > 1$), τ values are close to apparent operational efficacy τ' . Except for $\beta=2$, pK_A values are overestimated.

Figure 5 Homotropic allosteric modulation of efficacy

Dots, functional response to an agonist ($\tau=0.3$, $E_{MAX}=1$, $R_T=1$) acting at two sites with the same $K_A = 10^{-6}$ M exerting cooperativity in efficacy β indicated in the legend, according to Eq. 17. Full lines, left, Black & Leff equation (Eq. 7), right, Hill equation (Eq. 10) fitted to the data. Parameter estimates are in Table 3.

Table 3 Results of fitting Black & Leff and Hill equations to the model of homotropic allosteric modulation of efficacy

Black & Leff (Eq. 7) and Hill (Eq. 10) equations were fitted to model data (Eq. 17) with E_{MAX} fixed to 1.

Model Eq. 17 and 19				Black & Leff			Hill			
τ	pK_A	β	τ'	n	τ	pK_A	n_H	EC_{50}	E'_{MAX}	τ'
0.3	6.00	0.2	0.06	1.48	0.171	7.15	1.93	6.89	0.067	0.072
0.3	6.00	0.5	0.15	1.35	0.248	6.57	1.16	6.45	0.131	0.151
0.3	6.00	1.0	0.30	1.00	0.300	6.00	1.00	6.11	0.230	0.300
0.3	6.00	2.0	0.60	1.12	0.636	5.86	1.06	5.98	0.374	0.598
0.3	6.00	5.0	1.50	2.05	1.199	6.14	1.19	6.02	0.597	1.481

Fitting Eq. 17 with fixed system E_{MAX} to the model of functional response homotropic allosteric modulation of efficacy yields correct parameter estimates that are associated with the low level of uncertainty, except for neutral cooperativity ($\beta=1$) (Supplementary Information Figure S7). This should not represent a problem as for neutral cooperativity ($\beta=1$) Hill coefficient equals one and thus OMA in the form of Eq. 4 can be used.

OMA of substrate inhibition

Another common mechanism affecting the functional response to an agonist is substrate inhibition. In substrate inhibition, substrate concentration-dependently inhibits the reaction. In the case of functional response to receptor activation, agonist-receptor complexes [AR] are the substrate (Figure 6). Functional response is given by Eq. 21.

$$E = E_{MAX} \frac{[AR]}{K_E + [AR] \left(1 + \frac{[AR]}{K_I}\right)} \quad \text{Eq. 21}$$

Where K_I is the inhibition constant, the concentration of [AR] that causes 50% inhibition of the functional response. After substitution of Eq. 1 for [RA], Eq. 21 becomes Eq. 22 (Supplementary information Eq. A50).

$$E = E_{MAX} \frac{[A]\tau([A] + K_A)}{\sigma\tau[A]^2 + ([A] + K_A)^2 + [A]\tau(A + K_A)} \quad \text{Eq. 22}$$

Where $\sigma = R_T/K_I$. The apparent maximal response E'_{MAX} is given by Eq. 23 (Supplementary Information Eq. A46).

$$E'_{MAX} = E_{MAX} \frac{\tau}{1 + \tau(1 + \sigma)} \quad \text{Eq. 23}$$

Thus, apparent operational efficacy τ' is given by Eq. 24.

$$\tau' = \frac{\tau}{\sigma\tau + 1} \quad \text{Eq. 24}$$

The EC_{50} value for the model of substrate inhibition is given by Eq. 25 (Supplementary information Eq. A53).

$$EC_{50} = \pm \frac{\sqrt{\sigma^2\tau^2 + 4\sigma\tau^2 + 8\sigma\tau + \tau^2 + 16\tau + 8} - 2K_A\sigma\tau - K_A\tau}{2(\sigma\tau + 2\tau + 1)} \quad \text{Eq. 25}$$

The shape for functional response is similar to homotropic allosteric modulation of efficacy (Figure 6). Again, the Hill equation fits the data better than the Black & Leff equation. For strong inhibition ($\sigma=10$) curves of the functional response have a bell shape. Except for the bell-shaped curve, fitting of the Hill equation gives correct estimates of apparent maximal response E'_{MAX} and thus correct estimates of apparent operational efficacy τ' . In contrast, fitting the Black & Leff equation results in underestimated τ values and overestimated pK_A values. Stronger inhibition (greater value of σ) results in the larger overestimation of pK_A .

Figure 6 Substrate inhibition of functional response

Dots, functional response to an agonist ($\tau=0.3$, $E_{\text{MAX}}=1$, $R_T=1$, $K_A = 10^{-6}$ M) in the model of substrate inhibition according to Eq. 22. Values of σ are indicated in the legend. Full lines, left, Black & Leff equation (Eq. 7), right, Hill equation (Eq. 10) fitted to the data. Parameter estimates are in Table 4.

Table 4 Results of fitting Black & Leff and Hill equations to the model of substrate inhibition

Black & Leff (Eq. 7) and Hill (Eq. 10) were fitted to model data Eq. 22 with E_{MAX} fixed to 1.

Model Eq. 22 and 24				Black & Leff			Hill			
τ	pK_A	σ	τ'	n	τ	pK_A	n_H	EC_{50}	E'_{MAX}	τ'
0.3	6.00	0	0.300	1.00	0.300	6.00	1.01	6.00	0.226	0.300
0.3	6.00	0.5	0.283	1.02	0.292	6.05	1.01	6.15	0.221	0.283
0.3	6.00	1	0.231	1.14	0.276	6.25	1.06	6.26	0.188	0.231
0.3	6.00	5	0.120	1.37	0.218	6.76	1.37	6.63	0.109	0.121
0.3	6.00	10	0.075	1.37	0.159	7.04	1.72	6.87	0.073	0.079

Fitting Eq. 22 with fixed system E_{MAX} to the model of functional response of substrate inhibition yields correct parameter estimates that are associated with the low level of uncertainty (Supplementary Information Figure S8).

OMA of non-competitive inhibition

As shown in Figure 2, OMA with slope factor n allows for EC_{50} values higher than K_A . The simplest mode of interaction that increases observed EC_{50} above K_A is non-competitive autoin-

hibition. Under non-competitive auto-inhibition, AR non-competitively blocks functional response (Figure 7). It results in a concentration-dependent increase in EC_{50} and a decrease in E'_{MAX} . Functional response is given by Eq. 26.

$$E = \frac{E_{MAX}[AR]}{(K_E + [AR]) \frac{[AR]}{K_I + [AR]}} \quad \text{Eq. 26}$$

For $K_I > 0$, after substitution of Eq. 1 for $[RA]$, Eq. 26 becomes Eq. 27 (Supplementary information Eq. A57).

$$E = E_{MAX} \frac{\tau[A]}{K_A + [A](1+\tau)} \frac{\sigma[A]}{K_A + [A](1+\sigma)} \quad \text{Eq. 27}$$

Where $\sigma = R_T/K_I$. The apparent maximal response E'_{MAX} is given by Eq. 28 (Supplementary Information Eq. A57).

$$E'_{MAX} = E_{MAX} \frac{\tau}{1+\tau} \frac{\sigma}{1+\sigma} \quad \text{Eq. 28}$$

Thus, apparent operational efficacy τ' is given by Eq. 29.

$$\tau' = \frac{\sigma\tau}{\sigma+\tau+1} \quad \text{Eq. 29}$$

The EC_{50} value for the model of non-competitive auto-inhibition is given by Eq. 30 (Supplementary information Eq. A61).

$$EC_{50} = K_A \frac{\sqrt{\sigma^2 + 6\sigma\tau + 8\sigma + \tau^2 + 8\tau + 8 + \sigma + \tau + 2}}{2(1+\sigma)(1+\tau)} \quad \text{Eq. 30}$$

Non-competitive auto-inhibition decreases apparent maximal response E'_{MAX} , increases observed half-efficient concentration EC_{50} and results in steep curves (Figure 7). Resulting concentration-response curve is asymmetric with a typical slope factor about 1.2 regardless of values of K_I and K_E . Both models fit well. Fitting Hill equation gives correct estimates of apparent maximal response E'_{MAX} and thus correct estimates of apparent operational efficacy τ' (Table 5). In contrast, for $K_I \geq 1$, fitting the Black & Leff equation results in underestimated values of τ and pK_A .

Figure 7 Non-competitive auto-inhibition of functional response

Dots, functional response to an agonist ($\tau=0.3$, $E_{MAX}=1$, $R_T=1$, $K_A = 10^{-6}$ M) in the model of non-competitive autoinhibition according to Eq. 27. Values of inhibition constants K_I are indicated in the legend. Full lines, left, Black & Leff equation (Eq. 7), right, Hill equation (Eq. 10) fitted to the data. Parameter estimates are in Table 5.

Table 5 Results of fitting Black & Leff equations to the model of non-competitive auto-inhibition

Black & Leff (Eq. 7) and Hill (Eq. 10) equations were fitted to model data Eq. (27) with E_{MAX} fixed to 1.

Model Eq. 27 and 29				Black & Leff			Hill			
τ	pK _A	K _I	τ'	n	τ	pK _A	n _H	EC ₅₀	E' _{MAX}	τ'
0.3	6.00	0	0.300	1.00	0.300	6.00	1.00	6.11	0.231	0.300
0.3	6.00	0.5	0.182	1.44	0.307	6.02	1.20	5.87	0.153	0.181
0.3	6.00	1	0.130	1.38	0.230	5.94	1.21	5.81	0.114	0.129
0.3	6.00	2	0.083	1.32	0.154	5.87	1.22	5.75	0.076	0.082
0.3	6.00	5	0.040	1.25	0.077	5.8	1.22	5.70	0.038	0.040

Fitting Eq. 27 with fixed system E_{MAX} to the model of functional response of substrate inhibition yields correct parameter estimates that are associated with the low level of uncertainty only when correct initial estimates of τ and σ are given (Supplementary Information Figure S9 and S10). In the case of $K_I=5$ (Supplementary Information Figure S9), estimates of operational efficacy τ and inhibition factor σ are swapped pointing to the symmetry of Eq. 27. This symmetry makes calculation of τ and σ impossible as any τ and σ combination resulting in an appropriate apparent efficacy τ' (Eq. 28) fits well a given functional-response data (Supplementary Information Figure S10).

Discussion

The operational model of agonism (OMA)¹ is widely used in the evaluation of agonism. The OMA characterizes a functional response to an agonist by the equilibrium dissociation constant of agonist (K_A), the maximal possible response of the system (E_{MAX}) and the operational efficacy of agonist (τ) (Eq. 4). To fit non-hyperbolic functional responses slope factor n was introduced to the OMA (Eq. 7)². Analysis of the Black & Leff equation (Eq. 7) has shown that the slope factor n has a bidirectional effect on the relationship between the parameters E'_{MAX} and τ (Figure 1A versus 1C) and also affects the relationship between the parameters EC_{50} and K_A .

In practice, as exemplified in Figure 3, fitting the Black & Leff equation may result in operational efficacy τ lower than τ of control agonist although agonist exerts higher E'_{MAX} than control agonist. This may lead to wrong agonist ranking and compromise subsequent analysis and conclusions. Fitting Black & Leff equation to the models of homotropic allosteric modulation of affinity (Figure 4), homotropic allosteric modulation of efficacy (Figure 5), substrate inhibition (Figure 6) and non-competitive auto-inhibition (Figure 7) also resulted in wrong estimates of τ and K_A values (Tables 2 to 5).

Biased agonists stabilize specific conformations of the receptor leading to non-uniform modulation of individual signalling pathways⁸. To measure an agonist bias, the parameters τ and K_A must be determined and $\log(\tau/K_A)$ values of tested and reference agonist compared at two signalling pathways⁹. It is evident from the analysis of the Black & Leff equation, that as far as the EC_{50} value is dependent on parameters n and τ (Figure 1A vs. C), $\log(\tau/K_A)$ values cannot be compared to judge possible signalling bias unless the parameter n is equal to 1 for both tested and reference agonist.

Despite the dire effects of slope factor n , the Black & Leff equation is widely accepted^{7,10–16}. It even entered textbooks¹⁷. Very little concern on factor n has risen. For example, Kenakin et al.⁹ analysed in detail the effects of slope factor n on EC_{50} and τ to K_A ratio but did not deal with the bi-directional effect of n on τ neither proposed an alternative approach to avoid potential pitfalls. To force a proper shape on functional-response curves, Gregory et al.¹⁸ introduced the second slope factor into their OMA and operational model of allosterically modulated agonism (OMAMA) analysis making equations even more complex. So far, the greatest criticism of OMA was voiced by Roche et al.¹⁹, noting that to accommodate the shape of theoretical curves Black & Leff equation tends to overestimate equilibrium dissociation constant K_A and

operational efficacy τ and thus be misleading. They advocated for different expressions of operational models including OMA modified by Hill coefficient in the case of symmetric concentration-response curves.

The fitting of the Hill equation (Eq. 10) to the functional response is straightforward and easier than fitting the Black & Leff equation. As shown in Figures 3 to 6, the Hill equation fits well to various functional-response curves, often better than the Black & Leff equation. In contrast to the Black & Leff equation, the Hill equation gives correct estimates of maximal response to agonist E'_{MAX} and its half-efficient concentration EC_{50} as documented by Tables 1 to 5. In the case of the Hill equation, neither value of E'_{MAX} nor the value of EC_{50} is affected by the Hill coefficient (Figure 1B and D). Therefore, biased signalling may be inferred from the comparison of the ratio of intrinsic activity (E'_{MAX}/EC_{50}) of tested agonist to the intrinsic activity of reference agonist at two signalling pathways as in the case of Hill equation the E'_{MAX}/EC_{50} ratio is equivalent to τ / K_A ratio^{6,20}. As shown for experimental data in Figure 3 and Table 1, relative intrinsic activities reveal signalling bias even when calculation bias factor based on τ and K_A values estimated by Black & Leff equation gives an incorrect conclusion.

Further, if needed, apparent operational efficacy τ' can be calculated from known E'_{MAX} values and known maximal response of the system E_{MAX} . From the relationship between τ' and EC_{50} ^{4,21} the mechanism of functional response can be inferred by comparison to explicit models. Four examples of explicit models are given above. Subsequently, equilibrium dissociation constant K_A and operational efficacy τ alongside the rest of model-specific parameters can be obtained by fitting the explicit model to experimental data.

Conclusions

Analysis of the Black & Leff equation has shown that i) The slope factor n has a bidirectional effect on the relationship between the parameters E'_{MAX} and τ . ii) The slope factor n affects the relationship between the parameters EC_{50} and K_A . Fitting the Black & Leff equation gives wrong estimates of τ and K_A values when slope factor n differs from unity, limiting the use of the Black & Leff equation in the evaluation of signalling bias. Analysis of the Hill equation has shown that the Hill coefficient does not affect the relationship between the parameters E'_{MAX} and τ neither between the parameters EC_{50} and K_A . Fitting Hill equation to the concentration-response data gives good estimates of EC_{50} and E'_{MAX} values, which are suitable for further analysis of OMA, OMAMA and signalling bias, e.g. using relative intrinsic activities.

Methods

Preparation of cells and membranes

Spodoptera frugiperda cells (Sf9) (Gibco) were maintained as a suspension culture in serum-free insect cell growth medium SF900 III (Gibco) in a plastic Erlenmeyer flask in a shaking incubator at 27 °C and 135 rpm in a non-humidified environment. The cultures were maintained at a density of $1\text{--}4 \times 10^6$ cells/ml. The density of the cells was determined with a haemocytometer, and viability was assessed by the exclusion of 0.2% trypan blue (Sigma-Aldrich). Human M₂ receptors and α -subunits of G_{oA} and G_{i1} G-proteins were expressed in recombinant baculoviruses, which were constructed and generated according to Bac-to-Bac® Baculovirus Expression System manual (Life technologies)²².

100 ml of Sf9 cell suspension at a density of 2×10^6 cells/ml were co-infected with baculoviruses encoding the M₂ receptor and G_{oA} or G_{i1} at a multiplicity of infection MOI = 0.1: 0.1. All infections were allowed to proceed for 69 h. Infected cells were harvested by centrifugation at $500 \times g$ for 5 min and frozen at -80 °C.

The pellets of harvested cells were suspended in the ice-cold homogenization medium (100 mM NaCl, 20 mM Na-HEPES, 10 mM EDTA, pH = 7.4) and homogenized on ice by two 30 sec strokes using a Polytron homogenizer (Ultra-Turrax; Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen, Germany) with a 30-sec pause between strokes. Cell homogenates were centrifuged for 5 min at $1000 \times g$. The supernatant was collected and centrifuged for 30 min at $30,000 \times g$. Pellets were suspended in the washing medium (100 mM NaCl, 10 mM MgCl₂, 20 mM Na-HEPES, pH = 7.4), left for 30 min at 4 °C, and then centrifuged again for 30 min at $30,000 \times g$. The resulting membrane pellets were kept at -80 °C until assayed.

GTP γ S binding

Agonist stimulated [³⁵S]GTP γ S binding was performed as currently described²³. Briefly, it was performed in 96-well plates at 30°C in the incubation medium described above that was supplemented with freshly prepared dithiothreitol at a final concentration of 1 mM. Suspension of membranes of Sf9 cells expressing M₂ + G_{oA} or M₂ + G_{i1} were preincubated with GDP and agonists for 15 min, then [³⁵S]GTP γ S was added for an additional 20 min. The final concentration of GDP and [³⁵S]GTP γ S was 20 μ M and 500 pM, respectively. Nonspecific binding was determined in the presence of 1 μ M non-labelled GTP γ S. Incubations were terminated by filtration through GF/C filtration plates (Millipore) using a Brandel cell harvester (Perkin Elmer,

USA). Plates were dried in microwave oven 800W 3 min and then 40µl of ROTISZINT® Eco Plus (ROTH) was added. The plates were counted in the Wallac Microbeta scintillation counter.

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Acknowledgements

This research was funded by the Czech Academy of Sciences institutional support [RVO:67985823] and the Grant Agency of the Czech Republic grant [19-05318S].

Author contributions

JJ derived equation for theoretical models and wrote python code for model data analysis. AR and DN designed, conducted experiments and analysed experimental data. All authors contributed to writing the manuscript, read and approved final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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