Journal of Pharmacological and Toxicological Methods 83 (2017) 94–106

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Research article

Estimation of the receptor-state affinity constants of ligands in functional studies using wild type and constitutively active mutant receptors: Implications for estimation of agonist bias

Frederick J. Ehlert [a,b,*], Richard S.L. Stein [b]

[a] Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, CA 92697-4625, United States
[b] Department of Anatomy and Neurobiology, School of Medicine, University of California, Irvine, Irvine, CA 92697-4625, United States

ARTICLE INFO

Article history:
Received 14 May 2016
Received in revised form 17 September 2016
Accepted 27 September 2016
Available online 7 October 2016

Keywords:
Receptor state affinity constants
Constitutive activity
Agonist bias
System bias
Monod-Wyman-Changeux model
Observed affinity
Efficacy
Receptor activation
Active state
Inactive state

ABSTRACT

We describe a method for estimating the affinities of ligands for active and inactive states of a G protein-coupled receptor (GPCR). Our protocol involves measuring agonist-induced signaling responses of a wild type GPCR and a constitutively active mutant of it under control conditions and after partial receptor inactivation or reduced receptor expression. Our subsequent analysis is based on the assumption that the activating mutation increases receptor isomerization into the active state without affecting the affinities of ligands for receptor states. A means of confirming this assumption is provided. Global nonlinear regression analysis yields estimates of 1) the active (K_{act}) and inactive (K_{inact}) receptor-state affinity constants, 2) the isomerization constant of the unoccupied receptor ($K_{q\text{-}obs}$), and 3) the sensitivity constant of the signaling pathway ($K_{E\text{-}obs}$). The latter two parameters define the output response of the receptor, and hence, their ratio ($K_{q\text{-}obs}/K_E$) is a useful measure of system bias. If the cellular system is reasonably stable and the $K_{q\text{-}obs}$ and $K_{E\text{-}obs}$ values of the signaling pathway are known, the K_{act} and K_{inact} values of additional agonists can be estimated in subsequent experiments on cells expressing the wild type receptor. We validated our method through computer simulation, an analytical proof, and analysis of previously published data. Our approach provides 1) a more meaningful analysis of structure-activity relationships, 2) a means of validating in silico docking experiments on active and inactive receptor structures and 3) an absolute, in contrast to relative, measure of agonist bias.

1. Introduction

The high-resolution crystal structures of the β_2-adrenergic receptor in a complex with inverse agonist and with both agonist and G_s provide some of the most striking evidence for functional states of a G protein-coupled receptor (GPCR) (Rasmussen et al., 2007, 2011). These advances raise the question of how can the functional responses of GPCRs be analyzed to determine the affinity of drugs for receptor states?

The conventional approach for quantifying drug-receptor interactions involves measuring the parameters, observed affinity and relative efficacy. Efficacy (ε) represents the fraction of the population of ligand-receptor complexes in the active state, and the observed affinity constant, K_{obs}, the reciprocal of the concentration of ligand required for half-maximal occupancy of the receptor population (Furchgott, 1966; Furchgott & Bursztyn, 1967). For agonists, K_{obs} represents a weighted average value of the active and inactive receptor-state affinities (K_{act} and K_{inact}) (Monod, Wyman, & Changeux, 1965; Staus et al., 2016), and hence, it does not represent a measure of affinity for either state.

The relationships between affinity and efficacy and the underlying state parameters demonstrate that the product of the efficacy and observed affinity of an agonist (εK_{obs}) is proportional to the active state affinity constant (K_{act}) (Ehlert, 2015; Tran, Chang, Matsui, & Ehlert, 2009). Hence, if the εK_{obs} product of one agonist is divided by that of another, a relative estimate of K_{act} is obtained. This value was initially termed *relative intrinsic activity* (RA_i) (Ehlert, Griffin, Sawyer, & Bailon, 1999). Both null ($RA_i = \varepsilon K_{obs}/\varepsilon' K_{obs}'$) and operational ($RA_i = \tau K_{obs}/\tau' K_{obs}'$) methods of regression analysis have been developed to estimate RA_i from agonist concentration-response curves (Ehlert, 2008; Ehlert et al., 1999; Figueroa, Griffin, & Ehlert, 2008; Griffin, Figueroa, Liller, & Ehlert, 2007).

Because biased signaling involves the induction of a unique active receptor state, the RA_i value is useful for detecting agonist bias (Ehlert, 2008; Kenakin, Watson, Muniz-Medina, Christopoulos, & Novick, 2012; Tran et al., 2009). Its relative nature raises ambiguity as to which agonist is biased—the agonist of interest or the reference agonist to which the RA_i value is normalized. Ideally, the RA_i value of a test agonist is normalized relative to an agonist (e.g., natural ligand) that lacks

* Corresponding author at: Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, CA 92697-4625, United States.
E-mail address: fjehlert@uci.edu (F.J. Ehlert).

http://dx.doi.org/10.1016/j.vascn.2016.09.007
1056-8719/

selectivity, so that any difference in pathway RA_i values can be attributed to bias of the test agonist. Nonetheless, methods for estimating K_{act} and K_{inact} in units of M^{-1} would provide a better approach for quantifying agonist action, particularly in cases where a receptor has more than one natural ligand.

To extract these estimates from the functional responses of agonists, two problems need to be solved. First, the response to an agonist is usually measured at a point downstream in the signaling pathway, and hence, the relationship between receptor activation and response is undefined. This transducer function can be deduced (operational model, (Black & Leff, 1983; Black, Leff, Shankley, & Wood, 1985)) or eliminated from the analysis (null method, (Furchgott, 1966; Furchgott & Bursztyn, 1967)) by measuring agonist responses in the absence and presence of either partial receptor inactivation or reduced receptor expression.

The second problem is that to estimate receptor-state constants, the effect of perturbing the equilibrium between active and inactive states on the output response needs to be measured. We recently described a protocol for estimating K_{act} and K_{inact} that relied on an allosteric agonist to push the equilibrium in the direction of the active state (Ehlert & Griffin, 2014). The approach involves measuring agonist responses under conditions of allosteric agonism and in the presence of partial receptor inactivation or reduced receptor expression. A related approach has been described for ligand-gated ion channels based on an analysis of agonist-induced whole-cell current responses (Chang & Weiss, 1999).

The equilibrium between active and inactive states can also be altered to favor the active state by introducing a constitutively activating point mutation into a GPCR. This approach has also been used to estimate the K_{act} and K_{inact} values of agonists for ligand-gated ion channels (Auerbach, 2010; Jha & Auerbach, 2010).

In this report, we describe a protocol for estimating the K_{act} and K_{inact} values of orthosteric ligands from the functional responses of GPCRs heterologously expressed in cell lines. Our method involves measuring responses of a given GPCR and a constitutively active mutant of it. Agonist responses are measured in the absence and presence of either partial receptor inactivation or reduced receptor expression. Once this analysis has been completed for one agonist, the K_{act} and K_{inact} values of additional agonists can be estimated from their concentration-response curves measured using the wild type receptor. We validate our method analytically and with simulated data and apply our approach to the analysis of published data. We also describe an example of a scenario in drug discovery to illustrate how our approach can be used to discover biased agonists (see Discussion). Our method provides a powerful means of quantifying agonist bias, investigating structure-activity relationships, and validating in silico docking experiments on active and inactive receptor structures.

2. Methods

2.1. Simulation of agonist concentration-response curves

To validate and describe our method, we simulated agonist concentration-response curves and then analyzed the data to determine if we could estimate the receptor-state constants used to simulate the data. The simulations and analyses were done using Eqs. (4)–(6), and their derivation is described next.

We have previously shown that, with regard to G protein signaling, agonist-induced receptor activation is proportional to the formation of a quaternary complex consisting of the active state of the agonist-receptor complex (DR^*) associated with exchange state of the G protein (G^*) bound with GDP (DR^*G^*GDP) (Ehlert, 2008; Ehlert & Griffin, 2014; Stein & Ehlert, 2015). We have also shown that the function describing agonist-induced formation of this complex is consistent with a Monod-Wyman-Changeux model (Ehlert & Griffin, 2014). Hence, we used the following simplified form of the Monod-Wyman-Changeux model (one orthosteric binding site and no allosteric site; (Monod et al., 1965)) to simulate agonist-receptor activation:

$$T_f = \frac{1}{1 + \frac{DK_{inact} + 1}{K_{q\text{-}obs}(DK_{act} + 1)}} \tag{1}$$

In this equation, T_f represents the total fractional stimulus (constitutive and ligand-induced receptor activation), D, the orthosteric ligand concentration, $K_{q\text{-}obs}$, the observed isomerization constant of the unoccupied receptor, and K_{act} and K_{inact}, the active and inactive receptor-state affinity constants (units of M^{-1}). The isomerization constant ($K_q = R^*/R$) is a property of the free receptor and defines the spontaneous isomerization of the unoccupied receptor into the active state in the absence of ligands or any allosteric modulators (e.g., G proteins), whereas the observed isomerization constant ($K_{q\text{-}obs}$) describes the equilibrium between the active and inactive states of the unoccupied receptor in the presence of G protein and guanine nucleotides (Ehlert & Griffin, 2014).

To simulate a response downstream from receptor activation, we substituted Eq. (1) into the transducer function of the operational model (Black & Leff, 1983; Black et al., 1985),

$$response = \frac{M_{sys}}{1 + \left(\frac{K_{E\text{-}obs}}{T_f}\right)^m} \tag{2}$$

to yield an equation for the response to the agonist (Ehlert & Griffin, 2014):

$$response = \frac{M_{sys}}{1 + K_{E\text{-}obs}^m \left(1 + \frac{DK_{inact}+1}{K_{q\text{-}obs}(DK_{act}+1)}\right)^m} \tag{3}$$

In these equations, M_{sys} represents the maximum response of the signaling pathway for an agonist with infinite selectivity for the active state, m, the transducer slope factor, and $K_{E\text{-}obs}$, the observed sensitivity constant of the signaling pathway.

As described under "Results", our protocol involves measuring the responses of both wild type and constitutively active mutant receptors under control conditions and those of reduced receptor expression or partial inactivation. Thus, Eq. (3) can be modified to account for these additional variables (Ehlert & Griffin, 2014):

$$response = \frac{M_{sys}}{1 + \left(\frac{K_{E\text{-}obs}}{qB_{max\text{-}rel}}\right)^m \left(1 + \frac{DK_{inact}+1}{C_M K_{q\text{-}obs}(DK_{act}+1)}\right)^m} \tag{4}$$

In this equation, $B_{max\text{-}rel}$ represents the relative receptor density of the constitutively active receptor mutant, C_M, the scalar by which the isomerization constant of the mutant receptor is increased relative to that of the wild type receptor, and q, the residual fraction of the receptor population after reduced receptor expression or partial receptor inactivation with an irreversible inverse agonist. The mutant receptor must have the same K_{act} and K_{inact} values as the wild type, and a means of validating this requirement is described under "Discussion".

If an irreversible neutral antagonist is used to reduce the amount of orthosteric ligand binding sites, the receptor population behaves as two subpopulations – one unaffected by the irreversible ligand and the other having its orthosteric binding pocket blocked but not its constitutive activity. For this situation, the following equation applies (Ehlert & Griffin, 2014):

$$response = \frac{M_{sys}}{1 + \left(\frac{K_{E\text{-}obs}}{B_{max\text{-}rel}}\right)^m \left(\frac{q}{1 + \frac{DK_{inact}+1}{C_M K_{q\text{-}obs}(DK_{act}+1)}} + \frac{1-q}{1 + \frac{1}{C_M K_{q\text{-}obs}}}\right)^{-m}} \tag{5}$$