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PERSPECTIVE

Flapping Loops: Roles for Hinges in a Ligand-Binding Domain of the Nicotinic Receptor

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ABSTRACT

One of the goals of molecular pharmacology is to understand the machinery that converts information about the presence of a chemical (binding) to a functional consequence. Agonists are drugs that bind to their molecular targets and cause conformational changes underlying activation of the target. Inevitably, therefore, it can be difficult to disentangle the affinity of the agonist for the target from its efficacy in producing the ensuing conformational change. Efficacy depends on two factors: the intrinsic equilibrium between active and inactive states in the absence of agonist, and the energy contributed by the agonist as a result of the relative affinities of agonist for the active and inactive states. These difficulties are particularly frustrating when the goal is to determine the role(s) that particular residues in a target protein have in shaping the overall efficacy of a drug: how is it possible to unambiguously distinguish a role in determining intrinsic efficacy from one in determining relative affinity? This perspective highlights a research article in this issue (p. 351) that demonstrates a quantitative approach to the resolution of this problem in the case of activation of the muscle nicotinic receptor. This elegant (if demanding) approach involves determining, separately, the consequences of specific mutations on gating in the unliganded and liganded states.

Introduction

The evolution of our thinking about how drug effects are mediated received a strong impetus when Langley (1905) proposed that nicotine combines with a specific receptive substance in striated muscle (Fig. 1A) and shortly afterward, Hill (1909) derived a simple binding equation. However, further advances in understanding agonist activity were slow in coming. In 1956, Stephenson (1956) introduced the concept that "affinity," the binding interaction between drug and target, and "efficacy," the ability of the drug to produce an effect, are separable. del Castillo and Katz (1957) were the first to propose a specific kinetic scheme with separate steps for binding and activation (Fig. 1B), again for the muscle nicotinic receptor to explain the nature of partial agonists. Subsequent work demonstrated that almost all of the active nicotinic receptors had two bound agonist molecules. The advent of high-resolution single channel recordings then provided evidence that the receptors could be active, albeit rarely, when only a single agonist was bound (Colquhoun and Sakmann, 1985), or even in the absence of agonist (Jackson, 1984). At present, the core activation scheme for the nicotinic receptor shows six major states for the receptor: open- or closed-channel conformations, each with three degrees of ligation (Fig. 1C). Other long-lived states have been identified, most prominently the desensitized state, but they can be separated out in single channel recordings so that this core activation process can be studied. It is also clear that the transition between closed and open states proceeds through a series of short-lived intermediate states (Auerbach, 2010), some of which have been detected in high-resolution recordings (Lape et al., 2008; Mukhtasimova et al., 2009).

The scheme in Fig. 1C is a two-conformation, concerted transition mechanism (Wyman and Allen, 1951; Monod et al., 1965; Changeux and Podleski, 1968) in which the receptor can adopt two conformations—closed channel and open channel. The top row contains receptors with closed channels and corresponds to the low affinity conformation, with dissocia-

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ABBREVIATIONS: A, agonist molecule; R, receptor with a closed channel; R*, receptor with an open channel.

tion constant K_D. The bottom row shows receptors with open channels and corresponds to the high-affinity conformation (dissociation constant J_D, with $J_D \ll K_D$). Channel opening is indicated by vertical transitions between the two rows. Agonists activate the receptor by binding more tightly to the receptor with an open channel, thus increasing the proportion of receptors with open channels. There are two fundamental parameters in this scheme. The first is the opening equilibrium for the unliganded receptor, E_0, given by the ratio of the opening rate for the unliganded receptor to the closing rate (R ↔ R* in the figure). The second is the affinity for the closed state relative to that for the open state ($\lambda = K_D/J_D$). The efficacy for a diliganded receptor (E_2) is determined by both E_0 and λ ($E_2 = \lambda^2 E_0$). Accordingly, by determining E_0 and E_2, it is possible to calculate the ratio of affinities, λ.

The research in the article by Purohit and Auerbach (2011) is on the muscle nicotinic receptor. A long series of studies has provided a strong quantitative picture of activation, and the analysis of a myriad of mutated residues (in conjunction with structural information) has given insights into the timing of conformational changes in different regions, the interactions among amino acid side chains, and the basis for interactions between an agonist and the receptor. The authors have extended this knowledge by dissecting the contribution of particular residues to two aspects of agonist efficacy: the relative binding affinities for the closed- and open-channel states and the intrinsic efficacy for channel opening. The approach relies on the kinetic framework shown in Fig. 1C.

The agonist-binding site in the nicotinic receptor is formed at the interface between two subunits [see Fig. 1 in Purohit and Auerbach (2011)]. The α subunit contributes three "loops" (the A, B, and C loops) and the adjacent δ or ε subunit contributes three more. The α subunit B loop contains a critical tryptophan residue (Trp149) which is involved in agonist binding, and has 2 glycine residues, one at either end (Gly147 and Gly153). The question the authors address is whether flexibility of the loop, associated with presence of the glycine "hinges" at the ends, is required for normal function.

Purohit and Auerbach (2011) measured the opening equilibrium for the diliganded receptor (E_2) and for the unliganded receptor (E_0), using single0channel recordings. E_2 is (relatively) straightforward: the channel opening rate is measured at a saturating concentration of agonist and the closing rate at a low concentration, and the ratio is computed. Measuring E_0 is more complicated, because the value is extremely low (for wild-type receptors it is approximately 5×10^{-7}), and so events are too rare to unambiguously characterize. [Values for E_0 have been estimated for other channels and also are quite small; e.g., for the $GABA_A$ receptor $\sim 10^{-5}$ (Chang and Weiss, 1999) and for the BK potassium channel $\sim 10^{-7}$ (Horrigan and Aldrich, 2002).] Purohit and Auerbach (2011) used a subunit containing a set of mutations that greatly increase the occurrence of unliganded openings as the base construct. The effect of the additional mutation under study was then compared with the base parameters. This approach relies on a demonstration that the effects of the background mutations are independent (energetic contributions to activation are additive) among themselves and with the test mutation (Purohit and Auerbach, 2009, 2010; Jha and Auerbach, 2010).

What is the answer? Mutations to the two glycines have opposite effects on the efficacy of gating for diliganded receptors, by affecting different parameters (see Fig. 2). Mutations to Gly147 decrease E_0 by decreasing the opening rate and increasing the closing rate. In contrast, mutations to Gly153 increase E_0 by increasing the opening rate and somewhat slowing the closing rate. The consequences for the relative affinities are different. In this case, mutations to Gly147 greatly decrease λ, whereas mutations to Gly153 decrease λ only slightly. The overall effect is that the mutations to Gly147 greatly decrease the efficacy of gating for diliganded receptors (E_2 decreases more than 1000-fold), whereas mutations to Gly153 increase E_2 by 10- to 60-fold. The authors also measured the affinity for the resting receptor for se-

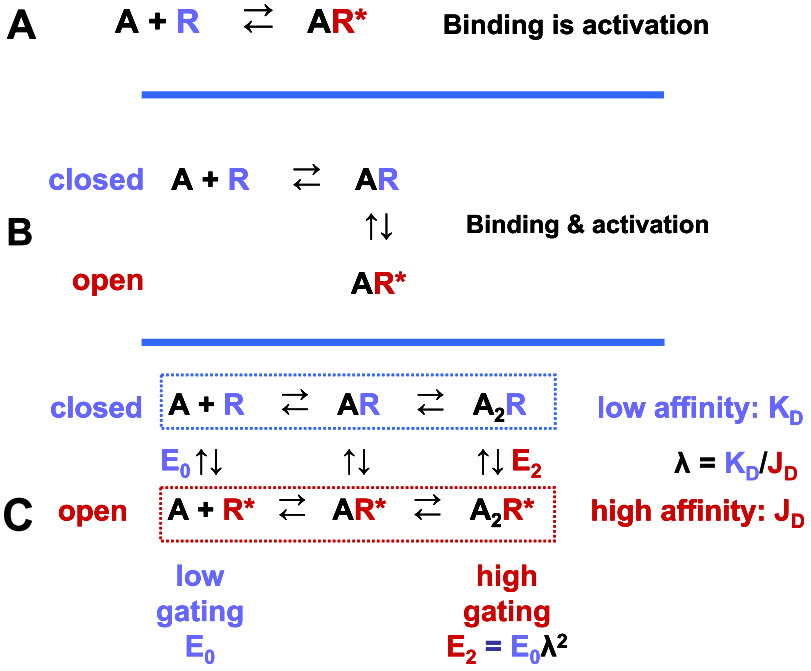

Fig. 1. Kinetic models for agonist activation of the nicotinic receptor. A, initial concept that an agonist (A) binds to a receptor (R), to produce an active complex (AR*; Langley, 1905; Hill, 1909). B, extension proposed by del Castillo and Katz (1957), in which binding and activation are separated into two steps. C shows the current scheme for the "core" activation process for the nicotinic receptor. There are two functional states for the receptor, that with a closed channel (boxed top row) with dissociation constant K_D and that with an open channel (bottom row) with dissociation constant J_D ($J_D \ll K_D$). Each functional state has three degrees of ligation with agonist, and the two binding steps for each functional form have the same microscopic affinities (they are independent and identical) (see Jha and Auerbach, 2010). Channel opening involves movement from the top row to the bottom row. Even in the absence of bound agonist (left-most column, R ↔ R*) opening can occur with an equilibrium open constant given by E_0 (E_0 = opening rate/closing rate). The fundamental parameters for the two-conformation, concerted transition model are shown. The first is the ratio of the dissociation constants for the closed (low-affinity) state to that for the open (high-affinity) state ($\lambda = K_D/J_d \gg 1$). The second is the intrinsic opening equilibrium for the unliganded receptor (E_0). The opening equilibrium for the doubly liganded receptor ($A_2R \leftrightarrow A_2R^*$; $E_2 = E_0\lambda^2$) is much larger than for the unliganded receptor.

lected mutations. The affinity was decreased by the mutation G147S (K_D increased), and increased by three mutations at Gly153. These "hinges" are clearly significant determinants of overall receptor function. Flexibility at Gly147 seems to be critical for positioning residues in the binding site for optimal interaction with the agonist, both for the resting receptor and even more so for the open-channel receptor. In contrast, flexibility at Gly153 seems to be more important for governing the efficacy of gating. As the authors put it, Gly147 is an "activation hinge," whereas Gly153 is a "deactivation hinge."

Mutations to the binding residue, Trp149, consistently reduce λ (Fig. 2) and have rather variable effects on E_0. These results indicate the importance of Trp149 in interacting with the agonist, particularly in the open state of the receptor. The effects on E_0 are an indication that structural changes in the ligand-binding domain (unsurprisingly) affect the overall conformational stability of the receptor.

Loop B has not been previously studied in terms of conformational flexibility. Attention has been focused on loop C because X-ray crystallographic studies of the related acetylcholine-binding protein indicated that loop C closes down on bound agonists (Celie et al., 2004), and studies of mutated subunits support the idea that this closure is functionally important (Mukhtasimova et al., 2009). The present work demonstrates that conformational flexibility in other parts of the binding site is critical for normal function of the receptor.

It is interesting to note that much larger changes occurred in the rate for entering the diliganded open state than in leaving the diliganded open state. This seems perhaps unintuitive, given that the high-affinity (open) conformation is expected to be stabilized by binding. The explanation probably lies in the rapid, largely unresolved transitions between states that the receptor makes as it opens (Auerbach, 2010). Perhaps the stabilizing energy contributed by agonist binding to the high-affinity form of the receptor increases the likelihood that the receptor will be in states further along the opening transition pathway, but before the final step for opening. This would agree with the observations that activation by agonists with different efficacies results in similar rates for leaving the diliganded open state but very different rates for entering it. Previous workers have also concluded that the major determinants of efficacy lie in transitions preceding actual channel opening (Lape et al., 2008; Mukhtasimova et al., 2009). One physical interpretation is that the increase in affinity reflects closure of the agonist-binding site (Mukhtasimova et al., 2009). In this case, it seems that flexibility in loop B is critical, as well as movement of loop C.

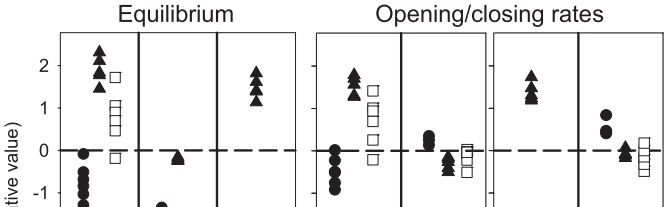

Fig. 2. Effects of mutations to the glycine "hinges" on kinetic parameters. The changes produced by mutations in parameters for activation are shown as the ratio of the values in the mutated receptor to the wild-type. Note that the ratios are plotted on a logarithmic scale, as the total range in ratios is approximately 10^7. The left shows relative values for the equilibrium parameters E_0, λ, and E_2 ($E_2 = E_0\lambda^2$). The right shows effects on rates (f_0 is the rate for entering the unliganded open state, b_0 the rate for leaving the unliganded open state, and f_2 and b_2 are the rates for the diliganded state). A change in E_2 ($E_2 = f_2/b_2$) is largely determined by the change in f_2. Values were obtained from the Supplementary Data in Purohit and Auerbach (2011).

This work sets a standard for examining structure-activity relationships for activation of proteins by drugs. It builds on a broad data base and requires the precision of single-protein kinetics, as well as the demonstration that a particular kinetic scheme is appropriate for analysis. The power of this approach is also a limitation: it requires the quantitative validation of the kinetic scheme applied and extremely high-quality data with high time resolution. The lessons learned will be valuable in interpreting data from other, less complete studies and will definitely be applicable to understanding the other members of the nicotinic receptor gene family.

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Wrote or contributed to the writing of the manuscript: Steinbach.

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