

## **2. CHARACTERIZATION OF RECEPTORS BASED ON RECEPTOR-MEDIATED RESPONSES**

The previous chapter described the evolution of the receptor concept and the early appreciation for the complexity that can exist between receptor occupancy and the ultimate physiological response. The present chapter summarizes methods used to characterize the specificity of a receptor-elicited response and strategies for determining the affinity constants of agonist, partial agonist, antagonist and inverse agonist agents at these receptors based on measurements of functional response.

### **CHARACTERIZATION OF RECEPTOR SPECIFICITY**

The analytical methods described had their origin in studies of receptor-mediated responses in native tissue. These same analyses, however, are useful for analysis of response in less complex preparations and, in fact, can even be applied to cell-based assays of heterologous receptor function in high throughput drug screens.

The very existence of receptors was predicted from numerous observations demonstrating the extraordinary specificity with which a response is elicited or antagonized when a series of drug homologs is evaluated in biological preparations. Consequently, it is the *specificity* of a drug or hormone action that persuades the investigator that an observed effect

is receptor-mediated rather than a nonspecific phenomenon independent of specific ligand-receptor interactions.

For endogenous stimuli or agonist drugs, the specificity of the putative receptor has been evaluated classically by determining the order of potency of a series of analogs in eliciting the desired response, e.g., contraction, secretion, ion or nutrient transport. Specificity is more easily demonstrated for small molecules than polypeptide hormones, because it is easier to prepare congeners with incremental modifications in substituent groups for small molecules. A classic example of using **order of agonist potency** in pinpointing a specific receptor's role is the pioneering work of Raymond Ahlquist (1948). Ahlquist demonstrated the existence of two receptor populations, now called  $\alpha$ - and  $\beta$ -adrenergic receptors, that mediate the physiological effects of the native catecholamines, epinephrine and norepinephrine. Ahlquist observed that catecholamines evoked smooth muscle contraction with an order of potency of norepinephrine > epinephrine > isoproterenol, and he termed these effects "alpha" ( $\alpha$ ). In contrast, Ahlquist noted that increases in cardiac chronotropy (rate) and inotropy (contraction) as well as smooth muscle relaxation were elicited by catecholamines with an order of potency of isoproterenol > epinephrine > norepinephrine. Ahlquist attributed these latter effects of catecholamines to a distinct population of adrenergic receptors, which he termed "beta" ( $\beta$ ). Thus, differing orders of agonist potency provided the original evidence for the existence of two adrenergic receptor populations, and remains the most common means for assessing receptor specificity today. It cannot be emphasized enough, however, that this method for comparing the relative potency of agonists is only useful if the agonists being studied possess the same efficacy, which often is not tested independently (see Furchgott [1972]; Kenakin [1987a]). An example of how agents with differing efficacies in different tissues can erroneously suggest the existence of distinct receptor populations is the reversal of the order of potency of oxymetazoline versus norepinephrine in eliciting  $\alpha_1$ -adrenergic receptor contractions in rat anococcygeus muscle compared to rat vas deferens. Because oxymetazoline is a partial agonist and these two tissues have a profound difference in "spare receptors" (i.e., relationship between occupancy and response), the order of potency of these two agents is reversed in these two tissues (Kenakin [1984b]). This example emphasizes that although the relative potency of agonists and partial agonists can be compared in a given tissue, it is unlikely that the dose-ratios for a pair of agonists in eliciting a particular response will be similar from tissue to tissue, since efficacy may vary from one target tissue to another due to differences in "receptor reserves" (i.e. receptor density, effector molecules and other modulators of the occupancy-response relationship).

The ability to assign multiple responses to a single, known receptor (encoded by a heterologously expressed cDNA) has revealed that the order of

potency of agonists, even when interacting with a single receptor population, can vary for different signal outputs if these agonists elicit or stabilize receptor conformations that have differing efficiencies in coupling to or activating these diverse signal outputs, e.g. GTP $\gamma$ <sup>35</sup>S binding, IP<sub>3</sub> production, Ca<sup>2+</sup> mobilization, secretion, and contraction (e.g. Berg et al. [1998]). These findings are troublesome for investigators trying to define the “specificity” of a receptor. Although differing orders of agonist potency for summated responses (e.g. contraction, secretion) in native tissues was classically *de facto* evidence for involvement of different receptors in eliciting response, studies with isolated cDNAs for a single receptor but measuring different signal outputs and/or monitoring agonists of differing efficacies for activating these outputs has revealed the inherent complexity in defining receptor specificity using agonist agents alone.

A second criterion of a specific receptor-mediated event is the **selectivity of blockade by antagonist agents**. For example, Ahlquist’s insightful proposal that distinct  $\alpha$ - and  $\beta$ -adrenergic receptors mediate catecholamine action was corroborated by later observations that  $\beta$ -adrenergic effects were selectively blocked by dichlorisoproterenol (later appreciated to be a partial agonist) and propranolol, whereas  $\alpha$ -adrenergic effects were selectively antagonized by phentolamine and phenoxybenzamine. Similarly, the subsequent subdivision of  $\beta$ -adrenergic receptors into  $\beta_1$ - and  $\beta_2$ -adrenergic receptor subtypes and  $\alpha$ -adrenergic receptors into  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptor subtypes was based primarily on the selectivity of different antagonists in blocking catecholamine effects in a variety of tissues (Schild [1973]; Berthelson and Pettinger [1977]). Unlike for agonists, the order of antagonist potency should be characteristic of a particular receptor whatever tissue preparation is employed. As discussed later, affinity constants for competitive antagonists, i.e. *null* antagonists with no intrinsic inverse agonist activity, are readily measurable (in contrast to those for agonists, partial agonists, and inverse agonists), and these constants can be helpful in classifying receptors. Based on receptor theory, it is expected that when different agonists interact with the same receptor population, the affinity constant calculated for a pure competitive antagonist should be the same regardless of which agonist is used to provoke the measured response. An assessment of whether the same  $K_I$  (or  $K_{D_B}$ ) for an antagonist is obtained in the presence of several agonists provides insight into which agonists converge on a common receptor population that also is recognized by the antagonist.

There are limitations, however, in concluding that two agents act via an identical receptor population if they mutually antagonize one another’s physiological responses. Thus, counter-regulatory effects mediated via distinct receptor populations are a fundamental mechanism by which a physiological steady state is maintained. These counter-regulatory effects

represent *functional* antagonism, although the agents involved elicit their effects through distinct receptors rather than by competing for occupancy of the same receptor population. For example,  $\beta$ -adrenergic effects on cardiac inotropy and chronotropy continually are countered by acetylcholine acting via muscarinic receptors. Catecholamines elicit their effects on cardiac function by elevating intracellular cAMP levels and regulating  $\text{Ca}^{2+}$  currents, whereas muscarinic agents decrease cAMP levels, suppress voltage-gated  $\text{Ca}^{2+}$  currents and activate hyperpolarizing  $\text{K}^{+}$  currents. If one tests the effects of acetylcholine on isoproterenol-stimulated cAMP accumulation in cardiac tissue, one would observe a concentration-dependent rightward shift of the isoproterenol concentration-response curve when acetylcholine is added to the incubation. This apparent competitive antagonism might lead the naive observer to conclude that acetylcholine is a  $\beta$ -adrenergic antagonist and counters the effects of epinephrine by competing for agonist binding at the  $\beta$ -adrenergic receptor recognition site. One line of evidence that confirms that isoproterenol and acetylcholine elicit opposing effects on signaling pathways via independent populations of receptors is the observation that propranolol blocks the effects of isoproterenol, but not those of acetylcholine, on cardiac cells. Conversely, the muscarinic antagonist atropine blocks the effects of acetylcholine but not those of isoproterenol on this system.

Two independent experimental approaches beyond order of agonist and antagonist potency have been useful for differentiating the receptor(s) involved in mediating particular biological effects: (1) studies of protection against irreversible receptor blockade by reversible agonists or antagonists, and (2) cross-desensitization experiments. These methods were particularly important in early characterizations of receptor properties in native tissues, and also rely on the specificity of the receptor in interacting with particular agonist and antagonist agents.

To exploit the strategy of **protection against irreversible receptor blockade** requires the availability of an irreversible agent that reacts chemically with the same receptor recognition site as does the agonist (or antagonist), and thereby inhibits receptor-mediated functions by decreasing the density of available receptors and not by modification of some other domain of the receptor molecule or by interfering with receptor-effector coupling. As a result of binding to the receptor site, the irreversible agent causes a persistent blockade of the receptor over the time-course of the experiment. If the irreversible agent is incubated with the test tissue in the presence of a reversible agonist or antagonist that interacts with the same recognition site(s) as the irreversible ligand, then the *rate* of receptor inactivation by the irreversible agent will be slowed by competition of the reversible and irreversible agents for receptor occupancy. In contrast, when particular reversible agonists or antagonists do not afford protection, the data are consistent with the interpretation that these agents do not interact with the

binding site modified by the irreversible antagonist. To assess whether a series of drugs can protect against receptor inactivation, a target tissue or cell preparation is incubated with an irreversible agent in the absence or presence of reversible agents for varying periods of time, after which the incubation is terminated by extensively washing the biological preparation. The extent of receptor inactivation that occurred during incubation with the irreversible agent is assessed by determining the extent to which an agonist can still elicit its characteristic effect in the treated preparation when compared with control preparations. In these studies, a control incubation with the protectant and *no* irreversible agent must be performed to permit assessment of whether the washing protocol used to terminate the incubation was sufficient to remove all of the protecting drug from the bathing medium, and of sufficient duration to permit dissociation of reversibly bound ligand from the tissue receptors. The most convincing evidence that reversible agonists or antagonists are interacting with (and thus protecting) the same receptor site inactivated by the irreversible ligand is that the presence of the reversible ligand decreases the *rate* of irreversible inactivation. Occasionally, when concentrations of reversible and irreversible agents are chosen appropriately, protection of the receptor by reversible agents may be apparent even at the longest interval of incubation with the irreversible antagonist. It should be remembered, however, that once the irreversible ligand occupies the receptor, the receptor binding site is inactivated and no longer vacant for occupancy by the protectant. Therefore, as the duration of incubation with the irreversible antagonist increases, the ability to detect protection against inactivation will decrease.

An example of the successful use of the protection approach to identify multiple receptor populations is a series of experiments performed by Furchgott (1954) using the  $\beta$ -haloalkylamine dibenamine as an irreversible antagonist. Furchgott demonstrated the probable existence of at least four independent receptor populations that evoked smooth muscle contraction in rabbit aortic strips. He conceptualized the protection experiments as either "self-protection" or "cross-protection." In self-protection experiments, the agonist present during incubation with the irreversible antagonist was the same agonist with which he subsequently assessed contraction. In "cross-protection" protocols, the agonist present during the receptor inactivation phase was different from that used to elicit contraction after extensive tissue washing. By definition, when reversible antagonists were used to protect receptors against irreversible blockade, the experimental design was one of "cross-protection." The only difference in the experimental protocol using agonists versus antagonists as the protectant is that the characteristically slower rate of antagonist dissociation from receptors requires a longer duration after washout of the protecting antagonist before retesting the agonist-elicited response. Using cross-protection studies, Furchgott

demonstrated that cross-protection occurred among norepinephrine, epinephrine and isoproterenol; he used this as evidence to conclude that these three agonists all acted on the same receptor, later defined as the  $\alpha$ -adrenergic receptor. By contrast, none of these catecholamines could afford cross-protection against inactivation of receptors for histamine, acetylcholine, or serotonin, and none of these latter agents protected among themselves or against inactivation of the catecholamine binding site. The above findings were taken together as evidence of the existence of distinct receptors for histamine, acetylcholine, and serotonin (in addition to those for catecholamines) that could mediate contraction of the rabbit aorta.

Protection against irreversible inactivation as a strategy for delineating multiple populations (or not) of receptors has limitations beyond the need for highly specific agents to serve as protectants. Thus, the existence of spare receptors in a tissue preparation also can give rise to confounding results. Even after a major fraction of a particular receptor population is inactivated, a high concentration of agonist may still elicit a full physiological response. This might lead an investigator to the erroneous conclusion that the irreversible antagonist was not interacting with the particular receptor under study. However, this potential limitation can be overcome by comparing the dose-response relationship for the agonist before and after multiple treatments with the irreversible antagonist that block increasing fractions of the putative receptor population. When a generous receptor reserve exists, irreversible receptor blockade of the "spare" receptors will first result in an increase in the  $EC_{50}$  for the agonist but no decline in maximal response, whereas progressive inactivation of the receptor population will ultimately result in a further increase in the  $EC_{50}$  and a decrease in the maximal response elicited by the agonist (cf. figure 2-1A).

A final experimental approach that has been used to delineate the specificity of the receptor population involved in a particular physiological response is that of **cross-desensitization** (Schild [1973]). Prolonged exposure to an agonist often results in a decline in the maximal response that can be elicited by that agonist. This agonist-induced decline in response has been referred to as *tachyphylaxis* or *desensitization*, and cross-desensitization studies exploit this property of agonists. Thus, if exposure to agonist A results in a decline in subsequent sensitivity to agonist A as well as to agonist B, but not to agonist C, then one interpretation of these findings is that A and B interact with a common receptor and C interacts with a distinct receptor population(s). This approach has been used successfully to demonstrate a multiplicity of functional receptors for opiates and opiate-mimicking peptides in the central nervous system (see Schultz et al. [1980]). There is a serious limitation to this approach, however, in that it assumes for its interpretation that the agonist utilized elicits only a "homologous" desensitization. Homologous desensitization occurs when an agonist interferes *only* with

physiological processes elicited by the particular receptor population with which that agonist interacts. In contrast, “heterologous” desensitization results when an agonist can desensitize a physiological response to subsequent stimulation by not only its own receptor but also by distinct receptor populations that activate the same response. For example, let us assume that  $\alpha$ -adrenergic, muscarinic cholinergic, and serotonergic receptors all elicit secretion of stored contents from a particular target tissue. If this tissue is exposed to an  $\alpha$ -adrenergic agonist for a prolonged period of time and becomes refractory to the addition of fresh agonist to the incubation, it has become desensitized. If addition of acetylcholine or serotonin still elicits the same extent of secretion characteristic of these agents in fresh tissue, then the catechoiamine-induced desensitization is said to be homologous, i.e., it only affects cellular activation via the  $\alpha$ -adrenergic receptor. If incubation with an  $\alpha$ -adrenergic agonist renders the tissue insensitive to acetylcholine and to serotonin as well, then the desensitization evoked by this agent is termed heterologous, as it affects not only  $\alpha$ -adrenergic but also muscarinic and serotonergic activation of the target cell, suggesting that molecular events downstream of the  $\alpha$ -adrenergic receptor, and shared by the muscarinic and serotonergic receptors, are involved in the desensitization process. Consequently, cross-desensitization experiments are only interpretable if the agonist used to induce tachyphylaxis evokes a homologous desensitization of the target tissue.

In summary, it is clear that each of the four experimental approaches outlined above for determining receptor specificity in native tissues is of potential value in assigning the specificity of a response to a particular receptor population. Because of the inherent limitations in each approach, however, the most definitive conclusions will result from combining some or all of these lines of experimental evidence. With the advent of molecular cloning, the specificity might be readily defined by expression of a single cDNA clone and defining specificity for a discrete signal transduction output, or comparing specificity for multiple outputs (Berget et al. [1998]). However, the existence of multi-subunit receptors, even for GPCRs, can sometimes confound the interpretations of even these seemingly straightforward experiments (Kuwasako et al. [2004]).

## **DETERMINING EQUILIBRIUM DISSOCIATION CONSTANTS ( $K_D$ VALUES) FOR RECEPTOR-LIGAND INTERACTIONS BASED ON MEASUREMENTS OF RECEPTOR-MEDIATED RESPONSE**

The determination of  $K_D$  values for receptor-ligand interactions from receptor-mediated response data involves multiple assumptions, or requirements of experimental systems. These assumptions were originally outlined by Furchgott (1966):

1. The response (e.g. of a tissue) should be due solely to the interaction of a hormone, neurotransmitter, or agonist drug with one type of receptor, and should not be a composite reflection of the effects of two receptor populations or secondary effects of an agonist, such as agonist-provoked neurotransmitter release or agonist-induced changes in blood flow, that might occur in an intact tissue preparation.
2. The altered sensitivity to an agonist observed in the presence of a competitive antagonist should be solely a result of competition between an agonist and antagonist for a shared recognition site.
3. The response obtained following addition of a given concentration of agonist should be measured at a time when the maximal response which that concentration of agonist can elicit has been reached. Similarly, allowing desensitization (or "fade") to occur will result in an underestimation of agonist potency. Biological preparations especially suitable for determination of the  $K_D$  values for receptor-ligand interactions are those that maintain a maximal level of response for a reasonable length of time, and do not manifest time-dependent desensitization or sensitization of the response. The experimental design always should include proper controls to permit measurement of, and thus correction for, any changes in sensitivity (e.g., desensitization or sensitization) to agonist during the time-course of the experiment.
4. When agonists or competitive antagonists are added to the incubation, the concentration of ligand free in solution should be maintained at a known level. Losses due to drug uptake or to chemical or enzymatic degradation of the ligand must either be prevented or overcome by continual re-addition of the appropriate concentration of ligand.



## Determination of $K_D$ Values for Receptor-Agonist Interactions, $K_{D_A}$

As might be expected, determining the equilibrium dissociation constant ( $K_D$ ) for binding of an agonist to its receptor using biological response data is not a straightforward procedure. If the assumptions of A. J. Clark were correct and tissue response was directly proportional to the fraction of receptors occupied, then the concentration of a hormone or an agonist drug that elicited a half-maximal response under steady-state conditions would be a direct measure of the  $K_D$  for agonist for formation of the agonist-receptor complex.<sup>1</sup> However, as described in chapter 1 (cf. figure 1-2) and emphasized in earlier sections of this chapter, maximal receptor-mediated effects can be elicited as a consequence of occupancy of only a small fraction of a total receptor population. Thus, the  $EC_{50}$  (concentration eliciting a half-maximal effect) for eliciting the biological response is often less than the  $K_D$  value for agonist-receptor interactions. As indicated in chapter 1, Stephenson formalized this nonlinear relationship between receptor occupancy and biological response by stating that the response of a tissue is some undefined function of stimulus  $S$ . He defined  $S$  as the product of the efficacy of an agonist,  $e$ , times the fraction of receptors occupied by the agonist:

$$\text{Response} = f(S) = fe \left( \frac{[RA]}{[R]_{TOT}} \right)$$

Based on the fundamental principles introduced by Stephenson, both Stephenson (1956) and Furchgott (1966, 1972) developed a method for determining the  $K_D$  value for agonists,  $K_{D_A}$ . Dose-response data for a particular agonist before and after irreversible receptor blockade are obtained. For certain receptor populations, a reasonably well-characterized drug may be available for this purpose, but for others an irreversible drug may not be

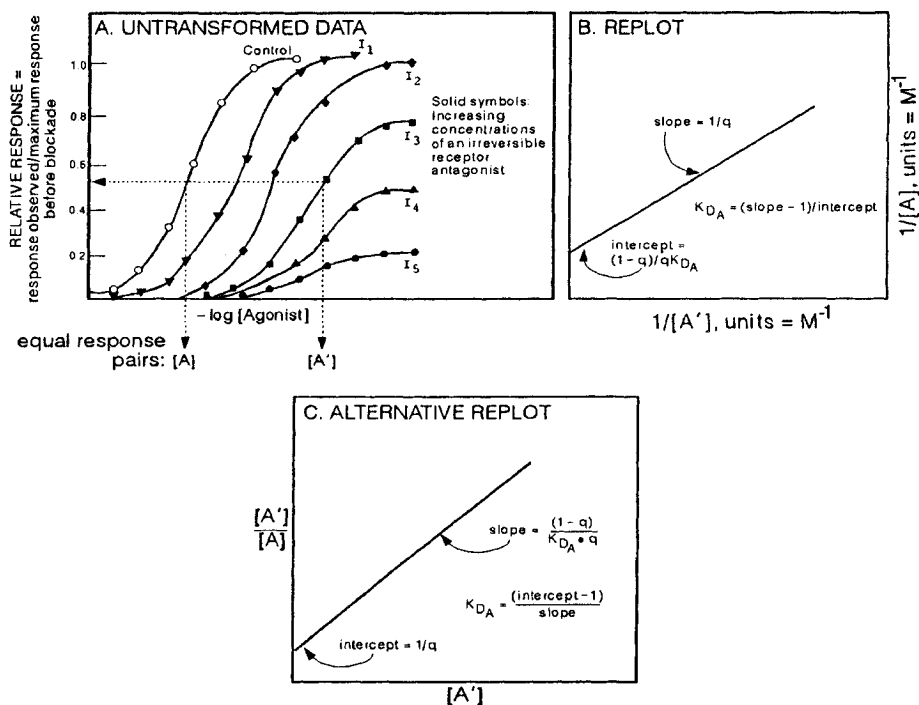
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<sup>1</sup> This textbook will maintain a uniformity of nomenclature throughout chapters 2–6. Although many originators of the algebraic relationships between concentration of agonist added and receptor occupancy attained refer to  $K_A$  as the equilibrium dissociation constant for agonist, this term is confusing, as the same term commonly is used to refer to equilibrium association constants, in units of  $M^{-1}$ . The term  $K_D$  always will be used to refer to equilibrium dissociation constants, in molar units. The  $K_D$  value for agonists will be denoted as  $K_{D_A}$ , for partial agonists as  $K_{D_P}$ , for antagonists as  $K_{D_B}$  and inverse agonists as  $K_{D_I}$ . There are several other examples where symbols used in original articles are changed in the mathematical descriptions summarized in the text, to emphasize the shared concepts inherent in the analyses and to minimize confusion caused by idiosyncratic nomenclature.

available. Crosslinking an antagonist ligand to a receptor using a bifunctional reagent might mimic the properties of a covalent ligand or, alternatively, blockade by an antibody directed against the binding domain(s) of the receptor might offer an alternative approach for incrementally decreasing the population of functional receptors on the cell surface. It may also be possible to inactivate receptor populations incrementally and irreversibly using reagents that covalently modify amino acid side chains. For example, several receptor populations appear to be sensitive to alkylation with N-ethylmaleimide, a sulfhydryl residue-directed reagent, or to reagents that modify the  $\epsilon$ -NH<sub>2</sub> group of lysine. Finally, it has been demonstrated that the equivalent of irreversible receptor blockade, i.e., irreversible receptor inactivation, can be accomplished by exposure of intact cells (Kono and Barham [1971]) or intact tissue (Lin and Musacchio [1983]) to proteases under conditions that do not alter the subsequent viability of the biological preparation.

Whether general or selective agents are employed to effect irreversible receptor blockade, the primary criterion for the validity of the procedure is that treatment must perturb only the receptor and not alter in any way the response system or receptor coupling to it. Once a method is in hand to decrease incrementally the concentration of receptor binding sites, agonist dose-response curves are obtained from control preparations and preparations in which receptors have been inactivated incrementally. For agonists possessing high efficacy (thus requiring occupancy of only a small fraction of the receptor population to elicit a maximal response), data obtained resemble those shown schematically in figure 2-1A. Thus, as an increasing fraction of the receptor population is inactivated, the agonist dose-response curve shifts to the right. Ultimately, the decrease in available receptors is sufficient to cause a decrease in the maximal response. For agonists possessing a lower efficacy (meaning that a larger fraction of the total receptor population must be occupied to elicit the maximal response), a shift to the right in the agonist dose-response curve may be subtle or not observed at all but may instead result in an immediately detectable decrease in the maximal response that can be elicited by the agonist.

The only assumption on which further analysis of the data shown in figure 2-1 is based is that an equal number of agonist-receptor complexes elicit equal responses, both before and after irreversible receptor blockade, i.e. the reagent used to effect receptor blockade does not also perturb the relationship between occupancy and response.



**Figure 2-1.** Determination of the  $K_D$  for receptor-agonist interactions ( $K_{D_A}$ ) utilizing the technique of irreversible receptor blockade. Dose-response relationships are determined before (control) and after exposure of tissue preparations to increasing concentrations ( $A$ ) of an irreversible antagonist,  $I_n$ . To obtain values for  $K_{D_A}$ , data can be replotted according to equation 2.7 (B), where  $q$  is the fraction of the receptor population remaining following irreversible receptor blockade, or according to equation 2.7A (C).

The algebraic relationships that result in the ability to determine the  $K_{D_A}$  for receptor-agonist interactions using experimental findings such as those shown in figure 2-1B are the following:

$$\frac{\text{observed response}}{\text{maximal response}} = \frac{E_A}{E_{\max}} = f(S) = fe \left\{ \frac{[RA]}{[R]_{\text{TOT}}} \right\} \quad (2.1)$$

The above equation is a restatement of the basic concept of Stephenson. However, Furchgott found it useful to modify this formula by introducing the term  $e$ , the **intrinsic efficacy**, where  $e = \frac{[R]}{[R]_{\text{TOT}}}$ . Therefore, substituting  $e = \frac{[R]}{[R]_{\text{TOT}}}$  into equation 2.1 yields:

$$\frac{E_A}{E_{\max}} = f \in \left\{ [R]_{TOT} \cdot \frac{[RA]}{[R]_{TOT}} \right\} = f \in \{[RA]\} \quad (2.2)$$

By introducing the term  $\epsilon$ , which has dimensions of the reciprocal of receptor concentration, Furchgott resolved the efficacy term of Stephenson ( $e$ ) into two components: the drug-dependent component  $\epsilon$  and the tissue-dependent component,  $[R]_{TOT}$ . This resolution of the two components inherent in drug efficacy emphasizes that Stephenson's efficacy term  $e$  is dependent on the total concentration of available, functional receptors,  $[R]_{TOT}$ , and that two different biological preparations containing the same response system will have different values for  $e$  depending on the extent to which  $[R]_{TOT}$  differs in the two biological preparations. This definition of  $e = \epsilon[R]_{TOT}$  and its substitution into the equations, however, does not affect the ultimate determination of  $K_{D_A}$  values.

Mass action relationships for a biomolecular interaction dictate that:

$$\frac{[RA]}{[R]_{TOT}} = \frac{[A]}{K_{D_A} + [A]} \quad (2.3)$$

Substitution into equations 2.1 and 2.2 yields:

$$\frac{E_A}{E_{\max}} = f(S) = fe \left\{ \frac{[A]}{K_{D_A} + [A]} \right\} = f \in \left\{ [R]_{TOT} \frac{[A]}{K_{D_A} + [A]} \right\} \quad (2.4)$$

If the treatment of a biological preparation with an irreversible antagonist reduces the concentration of total active receptors,  $[R]_{TOT}$ , to a fraction,  $q$ , of the original  $[R]_{TOT}$ , then the effective efficacy becomes  $qe$  or  $q \in [R]_{TOT}$ , and

$$\frac{E'_A}{E'_{\max}} = (S') = fe \left\{ q \frac{[A']}{K_{D_A} + [A']} \right\} = f \in \left\{ q[R]_{TOT} \frac{[A']}{K_{D_A} + [A']} \right\} \quad (2.5)$$

where  $E'_A$ ,  $E'_{\max}$ ,  $S'$  and  $[A']$  correspond to  $E_A$ ,  $E_{\max}$ ,  $S$  and  $[A]$ , respectively, following irreversible inactivation of  $[R]_{TOT}$  to  $q[R]_{TOT}$ . When comparing *equal responses*, i.e., when stimulus  $S$  before receptor inactivation with an irreversible antagonist is assumed to be equal to the stimulus  $S'$  after inactivation, then  $S = S'$  and, therefore,

$$\in [R]_{TOT} \cdot \frac{[A]}{K_{D_A} + [A]} = \in q[R]_{TOT} \cdot \frac{[A']}{K_{D_A} + [A']}$$

eliminating  $\in \cdot [R]_{TOT}$  yields

$$\frac{[A]}{K_{D_A} + [A]} = \frac{q[A']}{K_{D_A} + [A']} \quad (2.6)$$

The above mathematical manipulation has allowed cancellation of the receptor density term  $[R]_{TOT}$  and permits an estimate of  $K_{D_A}$  based entirely on response data. Note also that the method does not require knowing a value for  $f$ , the function which relates occupancy to response.

Equation 2.6 can be rearranged to the form:

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{(1-q)}{q \cdot K_{D_A}} \quad (2.7)$$

This is the linear transformation ( $y = mx + b$ ) shown in figure 2-1B. The slope of the line of a plot of  $1/[A]$  versus  $1/[A']$  is equal to  $1/q$  and the  $y$  intercept equals  $(1 - q)/qK_{D_A}$ . The  $K_D$  value for agonist,  $K_{D_A}$ , equals (slope - 1)/intercept. Furthermore,  $q$  (fraction of receptors remaining active) equals  $1/\text{slope}$ .

Equation 2.6 can be rearranged to a linear transformation other than that shown in equation 2.7. For example, by multiplying both sides of equation 2.7 by  $A'$ , one obtains:

$$\frac{[A']}{[A]} = 1/q + \frac{[A'] (1-q)}{K_{D_A} \cdot q} \quad (2.8)$$

A plot of  $[A']/[A]$  versus  $[A']$  yields a straight line with an upward slope. Statistical software tools can be used to provide estimates of the intercept and slope, from linear regression analysis, and the error of each of these two values. This line has a  $y$  intercept equal to  $1/q$  and a slope equal to  $(1 - q)/K_{D_A} \cdot q$ . The  $K_{D_A}$  value can be calculated as (intercept - 1)/slope. This alternative procedure for obtaining the  $q$  and the  $K_{D_A}$  value is shown in figure 2-1C. Furchgott (personal communication) favors the replot shown in figure 2-1C because data derived from studies at very low concentrations of agonist come at the beginning of the plot. Hence, these data do not significantly influence

the slope and intercept of the plot in figure 2-1C to the extent that they do in the double reciprocal plot in figure 2-1B. This is desirable because if there is any discrepancy between  $[A]_{\text{added}}$  and the *effective* concentration of agonist at the receptor because of tissue uptake or degradation, this discrepancy (i.e., error) would be greatest at very low concentrations of agonist.

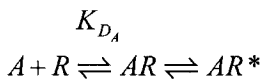
To transform the normalized dose-response data shown in figure 2-1A to the replots shown in figures 2-1B and 2-1C, *pairs* of concentrations of agonist resulting in *equal responses* before (i.e.,  $[A]$ ) and after (i.e.,  $[A']$ ) incubation with an irreversible antagonist are compared. Effective use of this technique requires that the dose-response curve after receptor inactivation has a depressed maximal response. Naturally, it is unlikely that experimentally added concentrations of agonist before and after irreversible receptor blockade will turn out to be pairs of  $[A]$  and  $[A']$  that elicit equal biological responses. Consequently, a curve must be drawn between the data points obtained, necessarily introducing some error into the subsequent analysis. The data analysis (and resultant determination of  $K_{D_A}$ ) is most practical and most valid statistically if the concentration pairs  $[A]$  and  $[A']$  are selected for replotting by starting with values of  $[A']$  actually added to the preparation exposed to an irreversible antagonist and then estimating the value of  $[A]$  that gives an equal response in the control preparation by inspecting the dose-response curve in the untreated tissue. The suggestion is practical, in that there is always a response ( $E_A$ ) in the control preparation that corresponds to a response ( $E_{A'}$ ) in the irreversibly inactivated preparation. However, the reverse will not always be true. The statistical validity of this approach is that the replot obtained using data pairs  $[A]$  and  $[A']$  can be analyzed more easily by least squares fitting, since  $[A']$  is determined experimentally (see Furchgott [1966], Parker and Waud [1971], and Thron [1973]). It also is worth reiterating that the analysis depicted in figure 2-1 requires only a single dose-response curve following irreversible receptor blockade for comparison with the control curve. However, confidence in the  $K_{D_A}$  value obtained is increased if the calculated value is similar when several levels of receptor inactivation are achieved as a result of incubation of the receptor-response system with various concentrations (or with a single concentration at various times) of an irreversible antagonist.

Certain assumptions were made in developing the theory for calculation of  $K_{D_A}$  following irreversible receptor blockade which must be met in the experimental protocol in order for the approach in figures 2-1B or 2-1C and the resultant analysis to be valid (Furchgott [1972]).

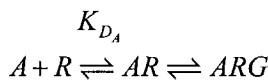
1. The agonist elicits its measured response by interacting with a single receptor population.

2. The interaction of agonist with the receptor is a reversible, bimolecular reaction operating according to the principles of mass action, such that  $[RA]/[R]_{TOT}$  can appropriately be equated with  $[A]/(K_{D_A} + [A])$  (cf. equation 2.3).
3. The receptor population involved is “uniform” with respect to  $K_D$  (this is inherent in the second assumption). Thus, it is assumed that neither negatively nor positively cooperative interactions occur among the receptors, so that the  $K_D$  of the receptor for agonists is independent of the degree of occupancy of the receptor population. This methodology does *not* apply if the receptor-response system is cooperative in nature (see Thron [1973]).
4. The relationship between stimulus  $S$  and concentration of agonist-receptor complexes  $[RA]$  (i.e., the intrinsic efficacy,  $\epsilon$ ) remains the same after irreversible inactivation of a part of the receptor preparation.
5. The  $[A]$  plotted as the “free drug” available to receptors is essentially equal to the concentration of drug added to the bathing solution or incubation medium.
6. Desensitization (or sensitization) to the agonist does not occur during the time-course of the experiments.
7. Inactivation of receptors is completely irreversible, such that the value of  $q$  does not change after washout of the irreversible blocker. Also, when evaluating the effects of an irreversible antagonist, the unbound antagonist should be removed by extensive washing of the preparations, so its concentration is essentially zero during subsequent measurements of response.

The development of experimental strategies to deduce the  $K_{D_A}$  for an agonist at its receptor based on agonist-elicited response data in a complex physiological system preceded methodologies for quantitating receptor density with radioligand binding or molecular insights into the biochemical events linking receptor occupancy to the ultimate physiological or pharmacological effect. However, as more molecular insights are gained about the receptor-elicited activation process, concern about the validity of the receptor inactivation method for determination of  $K_{D_A}$  has arisen (Leff et al. [1990] and references therein). For example, two tenable models for agonist activation of receptors are the **isomerization model**, e.g., for ligand-gated ion channels (del Castillo and Katz [1957]), and the **ternary complex model** and extensions of that model (cf. chapter 4) for receptors that control cellular processes via heterotrimeric GTP-binding proteins (DeLean et al. [1980]).

*Subset equations of the:**Isomerization Model*

Response

*Ternary Complex Model*

Response

In the isomerization model, agonist occupancy of the receptor evokes a conformational change in the receptor to an active state ( $R^*$ ), which is responsible for the receptor-elicited response. In the ternary complex model, the agonist-occupied receptor interacts with a heterotrimeric ( $\alpha\beta\gamma$ ) G-protein ( $G$ ) to facilitate GTP-binding to the  $\alpha$  subunit and activation of subsequent  $\alpha$  and/or  $\beta\gamma$  subunit-dependent responses. In *both* cases, if either  $AR^*$  or  $ARG$  accumulates, then the equilibrium will be pulled to the right, and the estimate of  $K_{D_A}$  will suggest that the receptor has a higher affinity for agonist than its intrinsic affinity would dictate. It can be shown for both the ternary complex and isomerization models that the estimation of affinity constants for partial agonists is subject to less error than estimations for full agonists, since these partial agonists convert only a small fraction of  $AR$  to  $AR^*$  or  $ARG$ . Despite the theoretical concern, however, only minimal errors in estimated affinity constants appear to have been made when a comparison of  $K_{D_A}$  values obtained in tissue-bath studies to those obtained using direct radioligand binding analyses (cf. chapter 3) has been undertaken (Leff et al. [1990]). Thus, classical pharmacological theory appears to offer experimental strategies that yield reasonable estimates of receptor affinity and, at the very least, provide an initial characterization of the properties of receptors based on the responses they elicit.

### **Determining $K_D$ Values for Receptor-Partial Agonist Interactions, $K_{D_P}$**

Multiple approaches for determination of the  $K_D$  values for partial agonists have been applied in the literature; three will be described in some detail here. The first is based simply on a comparison of the dose-response curve of a partial agonist to that of a full agonist (Barlow, Scott and Stephenson [1967]; Waud [1969]). The second approach determines doses of a full agonist that are equiactive in the absence and presence of a partial agonist (Stephenson [1956]; Colquhoun [1973]). A third strategy involves irreversible receptor



blockade of a sufficient fraction of the receptor population so that a partial agonist is no longer able to elicit any measurable biological response. The partial agonist is then used as a competitive antagonist of a full agonist, and its  $K_D$  for the receptor is determined as that for antagonists (Furchgott and Bursztyn [1967]). Each of these three approaches will be considered below. An alternative approach that will not be considered in detail here is that based on the model of Van Rossum and Ariëns for partial agonists (see Van Rossum [1963]), which evaluates the shift in the agonist dose-response curve caused by addition of increasing concentrations of a partial agonist to the incubation (see also Kenakin and Black [1978]).

The theory behind determining the  $K_D$  for receptor-partial agonist interactions by comparing the dose-response curves of partial agonists with those of full agonists is based on one principal assumption: that full or "strong" agonists elicit a maximal response by occupying only a small fraction of the total receptor population. Partial agonists, by definition, have a lower efficacy and must fill an appreciably greater fraction of the receptors to elicit a response. As described earlier, the observed response is some function of stimulus  $S = eY_A$ , where  $Y_A$  is the fraction of receptors occupied by the agonist. Fractional receptor occupancy  $Y_A$  can be expressed as:

$$Y_A = \frac{[RA]}{[R]_{TOT}} = \frac{[A]}{[A] + K_{D_A}}$$

The assumption is that for a full agonist,  $A$ , the  $[A]$  resulting in a response is very small relative to its  $K_D$  for interacting with the receptor. Thus, for a full agonist  $A$ :

$$Y_A = \frac{[A]}{K_{D_A}} \quad (2.9)$$

In contrast, for a partial agonist,  $P$ ,

$$Y_P = \frac{[RP]}{[R]_{TOT}} = \frac{[P]}{[P] + K_{D_P}} \quad (2.10)$$

and this relationship cannot be simplified further.

To determine experimentally the  $K_D$  for a partial agonist  $P$ , one obtains a dose-response curve for the full agonist  $A$  and the partial agonist  $P$ . Since, by definition,

$$S = e_A Y_A$$

$$S = e_P Y_P$$

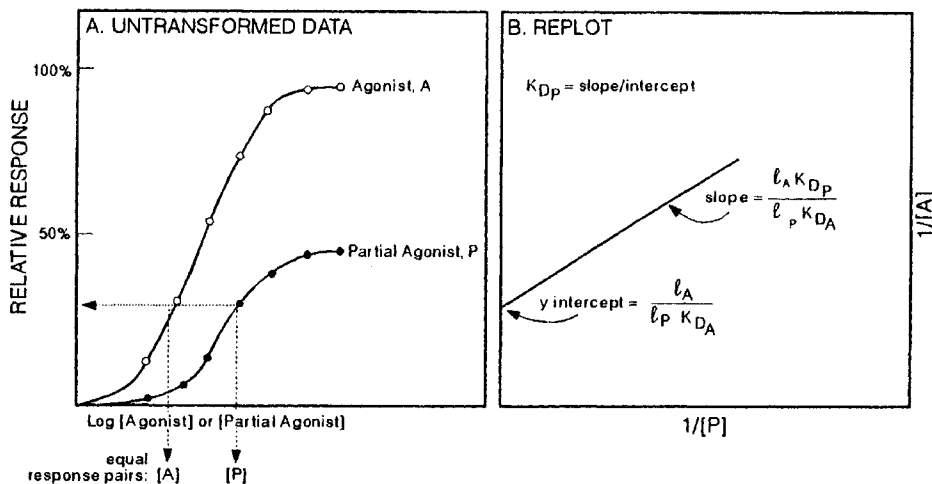
If one compares the concentrations of  $A$  and  $P$  that elicit *equal responses*, then

$$e_A \frac{[A]}{K_{D_A}} = e_P \frac{[P]}{[P] + K_{D_P}} \quad (2.11)$$

and by rearranging to a linear transformation one obtains:

$$\frac{1}{[A]} = \left[ \frac{e_A}{e_P} \cdot \frac{K_{D_P}}{K_{D_A}} \right] \frac{1}{[P]} + \frac{e_A}{e_P K_{D_A}} \quad (2.12)$$

Thus, a plot of  $1/[A]$  versus  $1/[P]$  should be linear if all assumptions regarding the ligand-receptor interactions are correct and a sufficient receptor capacity exists so that equation 2.9 is valid.



**Figure 2-2.** Determination of the equilibrium dissociation constant for a partial agonist,  $K_{D_P}$ , determined by comparison of dose-response curves of the partial agonist with a full agonist.

Figure 2-2A shows a schematic diagram comparing the response obtained with a full agonist and a partial agonist, and figure 2-2B shows the data transformation to obtain the equilibrium dissociation constant for the partial agonist  $P$ . The slope of the plot of  $1/[A]$  versus  $1/[P]$  equals

$\frac{e_A K_{D_A}}{e_P K_{D_A}}$ , and the intercept on the y axis is  $\frac{e_A}{e_P K_{D_A}}$ .

The equilibrium dissociation constant for partial agonist  $P$  can be calculated as follows:

$$K_{D_P} = \frac{\text{slope}}{y \text{ intercept}} = \frac{\left[ \frac{e_A}{e_P} \cdot \frac{K_{D_P}}{K_{D_A}} \right]}{\left[ \frac{e_A}{e_P} \cdot \frac{1}{K_{D_A}} \right]} = K_{D_P}$$

Furthermore, by knowing the equilibrium dissociation constant for the agonist  $K_{D_A}$  and setting an arbitrary value for the efficacy of the full agonist (e.g.,  $e_A = 1$ ), one can obtain the efficacy of the partial agonist ( $e_P < 1.0$ ).

It must be stressed that the assumption made in the method described above is that the concentration of agonist ( $[A]$ ) that produces a maximal response is much less than the equilibrium dissociation constant for the agonist  $K_{D_A}$ . If the tissue being studied does not have a receptor reserve for a full agonist, then an error term will be introduced into the calculation, such that the procedure will alter the estimate of the value for  $K_{D_P}$ :

$$K_{D_P} = \frac{\text{slope}}{y \text{ intercept}} \left( 1 - \frac{e_P}{e_A} \right)$$

It is apparent that the error diminishes to zero as the difference between the efficacy of the full and partial agonist increases (see Kenakin [1984a]).

In his initial paper which introduced his concept of efficacy, Stephenson suggested another method for estimating the  $K_D$  value for receptor-partial agonist interactions. It can be shown algebraically that an estimate of  $K_{D_P}$  can be made by determining doses of a full agonist that are equiactive in the presence ( $[A']$ ) and absence ( $[A]$ ) of a partial agonist  $P$ . Again, the assumption is made that the concentration of agonist eliciting a maximal response is much less than the  $K_D$  value for receptor interactions with "full" or "active" agonists. The equation that relates the equiactive doses of a full agonist in the absence and presence of a partial agonist is the following:

$$[A] = \frac{[A']}{1 + \frac{[P]}{K_{D_p}}} + \left[ \frac{e_p}{e_A} \cdot \frac{[P]}{K_{D_p}} \cdot \frac{K_A}{1 + \frac{[P]}{K_{D_p}}} \right] \quad (2.13)$$

and  $K_{D_p}$  is estimated from a plot of  $[A]$  versus  $[A']$  by the following relationship:

$$K_{D_p} = \frac{[P] \cdot \text{slope}}{1 - \text{slope}}$$

Again, if the assumption of a considerable spare receptor capacity for the “full” or “strong” agonist is not met, an error term of  $(1 - e_p/e_A)$  is introduced which diminishes to zero if  $e_A \gg e_p$  (see Kenakin [1984a] and references therein). An even more rigorous version of this method (Kauman and Marano [1982]) examines agonist concentration-response curves in the presence of a number of concentrations of the partial agonist, thus providing a range of partial agonist concentrations over which to estimate the  $K_{D_p}$  and utilizes slopes from a range of equiactive agonist concentration plots as:

$$\log \left( \frac{1}{\text{slope}} - 1 \right) = \log [P] - \log K_{D_p} \quad (2.14)$$

A plot of  $\log \left( \frac{1}{\text{slope}} - 1 \right)$  as the  $y$  axis versus  $[P]$ , log scale, yields a linear regression whose slope should not be significantly different from unity and whose intercept estimates the  $K_{D_p}$ .

A third technique for the determination of the  $K_D$  for a partial agonist ( $K_{D_p}$ ) was introduced by Furchgott and Burszty (1967) as an “internal check” for the value of  $K_{D_p}$  determined using the procedures described above. Incubation conditions for treatment with an irreversible antagonist are determined that still permit the effects of a strong or “full” agonist to be obtained but which inactivate a sufficient fraction of the receptor population so that the partial agonist under study no longer produces a response. The interaction of the partial agonist with its receptor can then be studied as a competitive antagonist of the full agonist. Consequently, the  $K_{D_p}$  under these circumstances can be estimated as for competitive antagonists (see following section):

$$K_{D_p} = \frac{[P]}{\text{dose ratio} - 1} \quad (2.15)$$

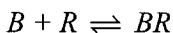
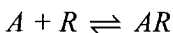
In some systems, partial agonists are of such low efficacy that a full agonist can still produce a further response. Experimentally, one observes an elevated baseline due to the partial agonist and a rightward shift of the control agonist-response curve, due to antagonism by the partial agonist. Dose ratios of equiactive concentrations of agonist can be estimated from the agonist-dependent region of the dose response curves. Kenakin (2004) has shown that a Schild analysis (see below) under such conditions will slightly underestimate the  $K_{D_p}$  value for the partial agonist, but the error will be minimal.

### Determining the $K_D$ Value for Receptor-Antagonist Interactions, $K_{D_B}$

An antagonist is any agent that blocks responses to agonist-evoked, receptor-mediated responses. Antagonists can act via the agonist-binding, so-called “orthosteric” binding pocket of the receptor, or they can suppress function as non-competitive inhibitors occupying other, allosteric sites. Orthosteric competitive antagonists, classically referred to as null antagonists, will cause rightward, parallel shifts in the agonist dose-response curve. Ultimately, the effects of the antagonist will be *surmountable* by agonist. Not all orthosteric antagonists, however, are devoid of intrinsic activity. As will be discussed in greater detail in chapter 4, some antagonists have a higher affinity for an active, rather than an inactive, state of the receptor; these agents can behave as partial agonists, particularly in receptor systems rendered constitutively active due to heterologous overexpression of a particular receptor. If antagonists have a higher affinity for the inactive state(s) of the receptor, then the antagonist will express inverse agonism, particularly in a constitutively active system. Where no agonist-independent activity occurs in a system, inverse agonists may appear to behave as simple competitive antagonists. The Schild analysis, explained below, was developed to quantitate the  $K_D$  for antagonist agents ( $K_{D_B}$ ) that were simple, competitive blockers at the orthosteric binding site.

When a strictly competitive, reversible antagonist interacts with a receptor population, its ability to influence receptor occupancy by an agonist is

determined both by the affinity of the receptor for the antagonist and the concentration of antagonist present. Thus, at the level of receptor occupancy, two independent equilibria are occurring for agonist  $A$  and antagonist  $B$ :



As a consequence of  $AR$  formation, a biological effect is elicited. In contrast, no effect is elicited as a consequence of  $BR$  formation when  $B$  is a null, competitive antagonist. Instead, fewer receptors are available for occupancy by the agonist. Competitive antagonists, therefore, suppress agonist-mediated responses by blocking access of the agonist to its specific receptor.

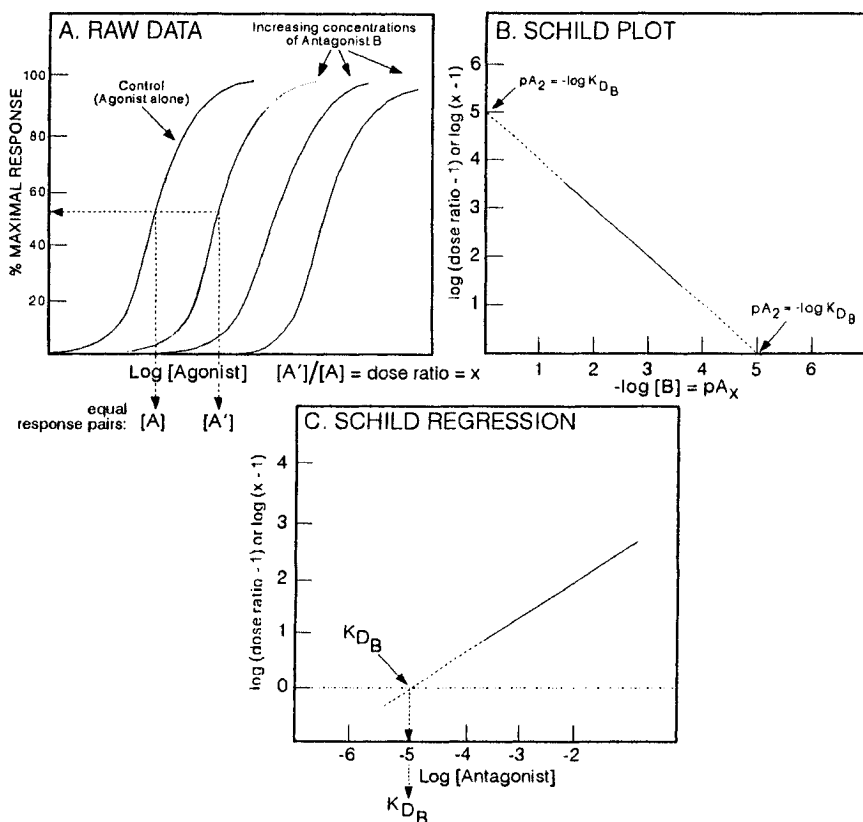
It was Gaddum (1937, 1943) who first formulated the relationship between the fraction of receptors occupied by an agonist as a function of the concentration agonist  $[A]$ , of competitive antagonist  $[B]$ , and of their respective equilibrium dissociation constants,  $K_{D_A}$  and  $K_{D_B}$ :

$$Y_{A'} = \frac{1}{1 + \frac{K_{D_A}}{[A']} (1 + [B]/K_{D_B})} = \frac{[A']}{[A'] + K_{D_A} (1 + [B]/K_{D_B})} \quad (2.16)$$

where  $Y_{A'}$  is the fractional receptor occupancy by the agonist that is diminished, due to the presence of antagonist, from the fractional occupancy  $Y_A$  obtained in the absence of antagonist, namely  $Y_A = [A]/([A] + K_{D_A})$ .  $[A']$  refers to concentration of agonist evaluated in the presence of antagonist.

As shown in figure 2-3A, the effects of competitive antagonists on agonist-provoked responses are evaluated by determining the dose-response relationship for agonist  $A$  in the absence and presence of increasing concentrations of an antagonist  $B$ . As indicated above, a series of parallel rightward curves is expected in the presence of increasing concentrations of a null, competitive, fully reversible antagonist.

Since the relationship between receptor occupancy by an agonist and the ultimate response is not necessarily linear, it is not possible to determine the  $K_{D_B}$  by fitting the data of a single agonist-dose-response curve in the presence of antagonist to equation 2.16. Instead, like methods described previously for



**Figure 2-3.** Determination of the  $K_D$  for receptor interaction with a competitive antagonist,  $K_{D_B}$ .

determining the affinities of receptor-agonist and receptor-partial agonist interactions, a “null method” is employed, i.e., one assumes that equal responses are elicited when an equal number of receptors are occupied by an agonist, such that

$$\frac{[A]}{[A] + K_{D_A}} = \frac{[A']}{[A'] + K_{D_A}(1 + [B]/K_{D_B})} \quad (2.17)$$

Schild simplified this equation to

$$\frac{[A']}{[A]} - 1 = \frac{[B]}{K_{D_B}} \quad (2.18)$$

This formulation permits the determination of  $K_{D_B}$  while making no assumptions regarding the relationship between fractional occupancy and the ultimate response. Schild denoted the ratio of agonist concentrations that elicits an equal response in the presence ( $A'$ ) or absence ( $A$ ) of antagonists as  $x$  (Schild [1957]). This ratio is often also referred to as the *dose ratio*. Furthermore, Schild defined  $pA_x$  as the negative logarithm of the antagonist concentration, in molar units, that produced the dose ratio of  $x$ . By taking the logarithms of equation 2.18 and substituting  $x$  for  $[A']/[A]$ , one obtains:

$$\log (x - 1) = \log [B] - \log K_{D_B} \quad (2.19)$$

Schild's definition of  $pA_x$  as  $-\log[B]$ , producing a dose-ratio of  $x$ , changes equation 2.19 to:

$$\log (x - 1) = -pA_x - \log K_{D_B} \quad (2.20)$$

It can be seen that when

$$x = 2, \log (2 - 1) = \log 1 = 0$$

Thus, when

$$x = 2, pA_x = -\log K_{D_B} \quad (2.21)$$

Consequently, a concentration of antagonist that shifts the agonist dose-response curve twofold is equal to the equilibrium dissociation constant for the receptor-antagonist interaction, i.e.,  $K_{D_B}$ . To determine graphically the value of  $pA_2$  from dose-response data such as those shown schematically in figure 2-3A, Schild introduced the plot shown in figure 2-3B, i.e.,  $\log (x - 1)$  versus  $-\log[B]$  from the linear transformation inherent in equation 2.20 (see Arunlakshana and Schild [1959]). Equation 2.20 shows that the intercept on the  $x$  axis ( $y = 0$ ) is equal to  $pA_2$ . Furthermore, since the theory inherent in derivation of the equation dictates that the slope of this plot must equal -1, the intercept on the  $y$  axis must *also* be equal to  $pA_2$ .

The transformation of data from a plot such as that shown in figure 2-3A to the **Schild plot** in figure 2-3B is straightforward. One obtains a family of dose-response curves for the agonist: a control curve in the absence of antagonist and agonist concentration-response curves in the presence of increasing concentrations of the putative competitive antagonist. When the control curve and a curve from an antagonist-containing incubation are compared, dose ratios are obtained, i.e., a ratio of the concentrations of agonist that elicit the *same response* in the presence ( $[A']$ ) or absence ( $[A]$ ) of



antagonist. (As mentioned above, the dose ratio =  $[A']/[A] = x$  of Schild.) The dose ratio theoretically can be taken at any response level that is not near the limits of the physiological response, where experimental error is too great. However, the dose ratio most commonly is compared at the  $EC_{50}$ , i.e., the concentration of agonist that elicits 50% of the maximal response. (Refer to figure 2-3A for determination of one pair of agonist concentrations,  $[A]$  and  $[A']$ ). To obtain the best estimate of  $pA_2$ , and hence  $K_{D_B}$ , it is useful to obtain  $\log(\text{dose ratio} - 1)$  values as close to zero as possible, i.e. using concentrations of antagonist that shift the agonist dose-response curve ~2-5 fold, ensuring that the experimental data cluster around the  $x$  intercept. Furthermore, a 30-100 fold range of antagonist concentrations should be used to obtain a statistically confident estimate of the slope (Kenakin [2004]).

A number of investigators quantitate receptor-antagonist interactions using the theory inherent in the Schild analysis but a slightly different data transformation from that of figure 2-2B. This transformation is sometimes called a **Schild regression** and is shown in figure 2-3C (Schild [1947]). Recalling equation 2.19:  $\log(x - 1) = \log[B] - \log K_{D_B}$ , the data can also be plotted as  $\log(x - 1)$  or the equivalent  $\log(\text{dose ratio} - 1)$  on the ordinate versus  $[B]$ , in log units, on the abscissa. The only difference in the plot is that there is a reverse sign of the slope, i.e., simple competitive antagonism should result in a straight line of slope = 1. The intercept on the  $x$  axis of a plot of  $\log(\text{dose ratio} - 1)$  versus  $\log[B]$  yields a direct estimate of the equilibrium dissociation constant for the antagonist,  $K_{D_B}$ .

The linear relationship between dose ratio and  $K_{D_B}$  results from the assumption that the antagonist interacts with a homogenous population of receptors at the orthosteric site for agonists with a single, unchanging affinity. Consequently, a Schild analysis yielding a straight line with a slope of 1 is consistent with the conclusion that the antagonist under study competitively antagonizes agonist occupancy of a homogenous population of receptors whose interactions with ligand is without positive or negative cooperativity. Since receptor affinity for the antagonist should be an unchanging parameter, the  $K_{D_B}$  value calculated for the antagonist should be independent of the agonist used, and Schild plots for multiple agonists studied in the presence of the same antagonist should yield indistinguishable  $K_{D_B}$  values.

Although the graphical method of Schild is straightforward, its application to raw data may nonetheless be complicated for either experimental or biological reasons. For example, if the antagonist-induced rightward shift of the agonist dose-response curve is not exactly parallel, then the points will not lead to a good linear fit (by non-weighted least squares linear regression analysis) and the slope of the best line may not equal -1 (figure 2-3B) or 1 (figure 2-3C). Interpretations of data when the slope  $\neq 1.0$

are given below. Furthermore, if there is considerable scatter around the line of slope = -1 or 1, the standard error of the intercept will be large, and there will be a corresponding large error in the estimate of  $K_{D_B}$ . The error of the extrapolation also depends on the distribution of data points: are they well spread along the line from its intercept at the  $y$  axis to its intercept at the  $x$  axis, or are the points clustered at one part of the line? The variability inherent in a poorly defined intercept (as is obtained with clustered data points) is immediately apparent when the  $pA_2$  value calculated from the  $x$  intercept differs significantly from that calculated at the  $y$  intercept of a Schild plot (figure 2-3B).

Several statistical approaches are available for analysis of Schild plots whose slope values are precisely -1.0 or 1.0 (see Parker and Waud [1971]; Mackay [1966;1978;1982]; Tallarida et al. [1979]). Perhaps the simplest approach to assure a reasonably reliable estimate of  $pA_2$  is to constrain the fit of the line so that the slope does equal -1 (figure 2-3B) or 1 (figure 2-3C), as theory dictates it should, and determine the  $pA_2$  value from the line best fitted by this slope value. This approach, however, is only tenable if the experimental data generate a line whose derived slope is not significantly different from 1.0, as determined by linear regression.

Schild plots that do not have a slope of 1 may reflect more complex receptor-antagonist interactions or, perhaps, technical limitations. Inadequate antagonist equilibration times prior to the study of agonist dose-response curves also will result in nonlinear Schild plots with portions possessing slopes  $> 1$  (Kenakin [2004]). It can take considerable time for agonist to equilibrate with receptors in the presence of antagonist, due to the characteristically slow dissociation rate of antagonists (Kenakin [2004], figure 6.14 and accompanying text). Similarly, if an uptake or enzymatic removal process is occurring such that the  $[A]$  added to the incubation is not the  $[A]$  available for receptor occupancy, then the *control* agonist concentration response curve will be shifted to the right of the true curve. Furthermore, if the antagonist blocks this uptake process at certain concentrations, the ultimate effect of these "side reactions" will be the appearance of even more complex Schild plots. The influence of agonist-uptake processes on Schild analysis has been dealt with quantitatively by Furchgott (1972) and Kenakin (1984a). Nonlinear Schild analyses also can arise as a result of any antagonist-produced effect independent of receptor blockade that potentiates response to the agonist. For example, if an antagonist not only blocks receptor occupancy by agonist but also results in the release of endogenous agonist, nonlinear Schild plots will result. Similarly, if an agonist is linked to stimulation of cAMP accumulation and the receptor antagonist not only blocks the receptor but also inhibits phosphodiesterase activity, the amplification of response due to the blockade of cAMP hydrolysis will result in nonlinear Schild plots. The

limitation of Schild analysis for studying antagonists with more than one site of function can be addressed using “resultant plots,” which are described later in this chapter.

The ability of Schild plots to reveal biological complexity, once technical contributors to non-linear Schild plots or plots with slopes  $\neq 1.0$  have been excluded, is of considerable value. Regressions with slope values  $< 1.0$  are characteristic of receptor heterogeneity. When agonist-dependent Schild plots provide evidence for the existence of receptor heterogeneity, such as receptor subtypes (cf. Furchgott [1978] and Kenakin [1987b]), the apparent receptor heterogeneity manifested by Schild analysis will be influenced by the relative concentration of receptor populations, the relative affinity of these populations for various agonists and antagonists, and by the effectiveness of coupling of these receptor populations to the measured response. Furthermore, the ratio of the concentrations of various receptor populations (i.e.,  $[R]_{TOT}$ ) and thus agonist efficacy at these receptors ( $e = \epsilon/[R]_{TOT}$ ) probably will vary from tissue to tissue. Consequently, the  $pA_2$  values obtained when receptor heterogeneity exists are *not* equivalent to values for  $K_{D_B}$ , but represent a composite of all of the above influences on apparent antagonist potency.

Although Colquhoun (1973) has shown that Schild analysis can be appropriate for various cooperative models of ligand-receptor interactions, important exceptions exist where Schild analysis cannot give a precise estimate of  $K_{D_B}$ , e.g., when two molecules of agonist must bind to two cooperatively linked sites for receptor activation to occur (Sine and Taylor [1981]). An example is the nicotinic cholinergic receptor on skeletal muscle linked to  $Na^+$  channel opening. In this case, the  $pA_2$  value calculated from a Schild analysis does not correspond to the  $K_{D_B}$  for receptor-antagonist interactions.

## Determining the Equilibrium Dissociation Constant for Inverse Agonists

When biological systems lack a “basal” or agonist-independent receptor activity, inverse agonists will produce parallel-rightward shifts in agonist-dose-response curves, i.e. will appear as null, competitive antagonists. In these situations, the  $K_D$  for the inverse agonist can be determined using Schild analysis, as described above. This is in fact why the inverse agonist properties of many antagonist drugs were not appreciated in native biological tissues or other preparations where agonist-independent, or constitutive, activity was subtle or non-existent.

Heterologous overexpression of cloned receptors, however, often results in spontaneous, agonist-independent activity of these receptors and an elevation of the baseline signaling response. Antagonists with negative

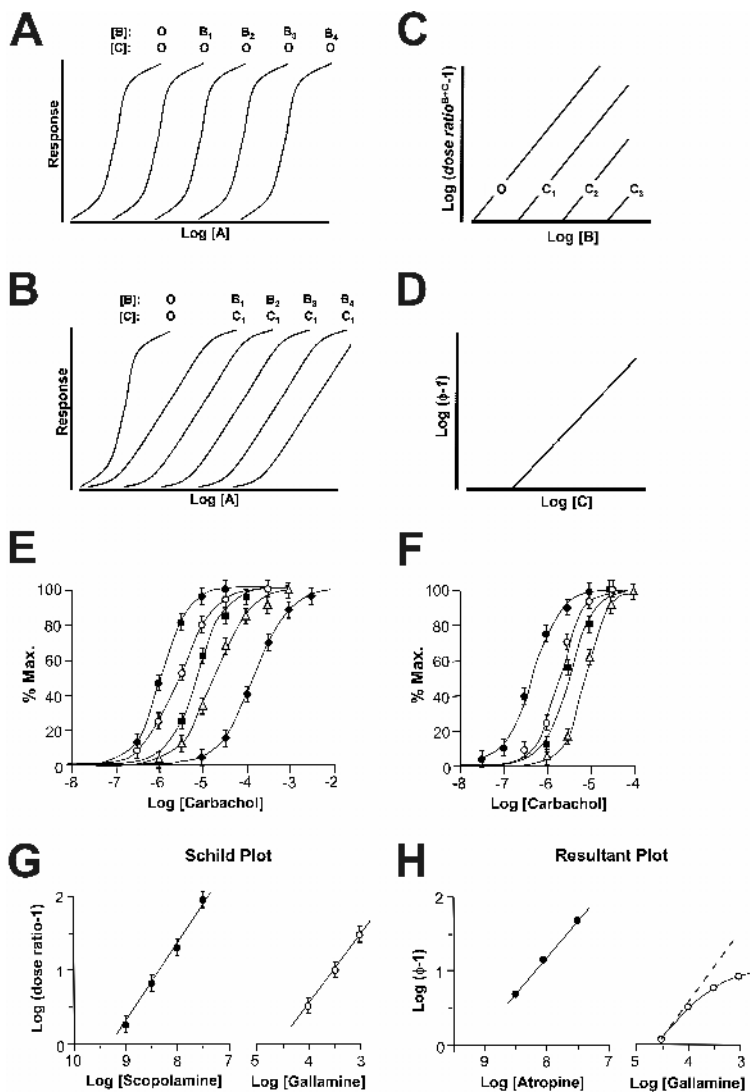
intrinsic activity do not correspond to the assumptions inherent in the Schild analysis of antagonist effects on agonist-elicited responses, i.e. there will be non-parallel shifts in the dose-response curves as “basal” or constitutively active receptor signal is reduced from baseline simultaneous with a competition for agonist-evoked response. Inverse agonists, by definition, will have a higher affinity for the inactive state of a receptor (cf. two- or more state models in chapter 1 and the extended ternary complex model for G protein-coupled receptors in chapter 4; Schutz and Freissmuth [1992]; Lefkowitz et al. [1993]). This is manifested by a dose-dependent decrease in the “basal” receptor activity by inverse agonists. Kenakin (2004) has shown that the concentration of inverse agonist that decreases basal receptor activity by 50% often approximates the concentration that produces a two-fold shift to the right of the agonist dose-response curve. Intuitively, however, the ability to estimate the  $K_{D_B}$  value for an inverse agonist from its impact on basal or on agonist-evoked receptor responses will be perturbed the most for agents that have profound differences in affinity for the active versus inactive states of the receptor. Further discrepancies between  $EC_{50}$  values for decreasing basal activity or for causing two-fold rightward shifts in agonist dose-response curves and the true  $K_{D_B}$  value for inverse agonists occur in highly coupled occupancy-response systems, again characteristic of heterologous overexpression systems. The observed  $EC_{50}$  will, in fact, be greater than the  $K_{D_B}$  in highly coupled systems.

## PHARMACOLOGIC RESULTANT ANALYSIS

Black (1986) initially introduced the method of pharmacologic resultant analysis to allow an estimation of the  $K_{D_B}$  for an antagonist that is known to have additional non-receptor effects, e.g. an antagonist that both blocks a receptor and, at higher concentrations, blocks neurotransmitter transport so that it alters neurotransmitter availability in the same preparation. However, as a general tool, performing resultant analysis provides further insight into the mechanism of action of an antagonist, i.e. whether it is acting as a pure competitive antagonist at the agonist-binding site or behaving as a modifier of agonist action via additional mechanisms.

Resultant analysis compares the blockade of agonist responses by two antagonists, one defined as the “reference antagonist,” a known competitive antagonist, and the “test antagonist,” which may have additional effects in addition to or independent of competitive antagonism. This method is similar to the additive dose ratio method of Paton and Rang (1965) to determine competitiveness, but is superior because it compensates for any secondary

effects of the test antagonist by determining both the “control” dose-response curve and the curve for evaluating the reference antagonist in the presence of the test antagonist, thus nullifying any non-receptor effects of the test antagonist.



**Figure 2-4.** Pharmacologic resultant analysis of competitive and allosteric ligands. The derivation of  $\phi$  and the properties of the Schild versus the resultant plot are discussed in the text. Panels A-D are modified from Black et al. (1986) and panels E-F are modified from Kenakin and Boselli (1989).

Resultant analysis is performed in the following manner (Black et al. [1986]); Kenakin [2001;2004]; and figure 2-4). The properties of a reference antagonist, denoted “ $B$ ,” known to be a competitive antagonist and presumably free from secondary, or “resultant,” activity, are compared to those of a test compound, denoted as “ $C$ ,” which is suspected of giving a blocking effect resulting from competitive antagonism plus additional actions. First, the response to agonist is examined alone, and then in the presence of the test antagonist,  $B$  (figure 2-4A). In a second series of experiments, the agonist is examined in the presence of a single concentration of the reference antagonist ( $C_1$ ) and the same increasing concentrations of the test antagonist,  $B$  (figure 2-4B). This second series is repeated for different concentrations of  $C$  ( $C_1, C_2, C_3$ ) to produce a series of “Schild lines,” i.e.  $\log(\text{dose ratio}^{B+C}-1)$  (figure 2-4C). The distance between each displaced Schild line and the control plot ( $C=0$ ) on the  $\log[B]$  axis is measured and defined as  $\log(\phi)$ . Replot values of  $\phi$  for different concentrations of  $C$  are then performed (figure 2-4D). If  $C$  is competitive or has a competitive property among other independent properties, then  $y=1+[C]/K_{D_C}$  and a plot of  $(\phi-1)/\log[C]$  will yield the  $K_D$  for  $C$  ( $K_{D_C}$ ).

The derivation of the equations, and the assumptions therein, are as follows. In simple competitive antagonism:

$$\text{response}^B = f \frac{[A^B]}{K_{D_A} \left( 1 + \frac{B}{K_{D_B}} \right) + [A^B]} \quad (2.22)$$

where response in the presence of a competitive (i.e. “reference”) antagonist,  $B$ , is shown by the Gaddum equation, and  $[A^B]$  signifies the concentration of agonist,  $A$ , evaluated in the presence of the test antagonist,  $B$ . By definition, the complex relationship between occupancy and response, denoted  $f$ , is not changed by  $B$ , allowing the cancellation of this function in the comparison of agonist response in the absence and presence of  $B$ , as described in equation

2.18 ( $\text{dose ratio} = 1 + \frac{[B]}{K_{D_B}}$ ), for the Schild equation. However, blockers of

receptor response not only may be competitive antagonists at the orthosteric

site but also may be allosteric in nature, and may change the relationship,  $f$ , between occupancy and response. Black et al. (1986) noted that when a ligand,  $C$ , both competes with  $A$  and alters the transducer function,  $f$ , then equation 2.22 is modified as follows:

$$\text{response}^C = f^C \left( \frac{[A^C]}{K_{D_A} \left( 1 + \frac{[C]}{K_{D_C}} \right) + [A^C]} \right) \quad (2.23)$$

When equal responses to  $A$  are observed in the absence and presence of  $C$ ,  $f$  and  $f^C$  cannot cancel. The solution of equation 2.23, however, comes from the additive rule of Paton and Rang (1965) for two null, competitive antagonists acting at the orthosteric site for agonist:

$$\text{response}^{B+C} = f \frac{[A^{B+C}]}{K_{D_A} \left( 1 + \frac{[B]}{K_{D_B}} + \frac{[C]}{K_{D_C}} \right) + [A^{B+C}]} \quad (2.24)$$

Equation 2.24 represents the case when  $B$  and  $C$  are both competitive antagonists. However, if  $C$  produces a “resultant” effect of competitive antagonism plus other effects, then equation 2.24 is modified to:

$$\text{response}^{B+C} = f^C \frac{[A^{B+C}]}{K_{D_A} \left( 1 + \frac{[B]}{K_{D_B}} + \frac{[C]}{K_{D_C}} \right) + [A^{B+C}]} \quad (2.25)$$

The relationship between occupancy and response in the presence of  $C$  does not cancel if one is comparing equation 2.22 with equation 2.25; it *does* cancel when comparing 2.23 and 2.25. Consequently for comparing equal responses of agonist in the presence of  $C$  versus  $C + B$ ,  $\text{response}^C = \text{response}^{B+C}$  and:

$$f^C \left( \frac{[A^C]}{K_{D_A} \left( 1 + \frac{[C]}{K_{D_C}} \right) + [A^C]} \right) = f^C \left( \frac{[A^{B+C}]}{K_{D_A} \left( 1 + \frac{[B]}{K_{D_B}} + \frac{[C]}{K_{D_C}} \right) + [A^{B+C}]} \right) \quad (2.26)$$

and with elimination of  $f^C$  and rearrangement:

$$\text{dose ratio}^{B+C} = \frac{[A^{B+C}]}{[A^C]} = 1 + \frac{[B]}{K_{D_B} \left( 1 + \frac{[C]}{K_{D_C}} \right)} \quad (2.27)$$

where dose ratio<sup>B+C</sup> is the concentration ratio of agonist,  $A$ , required to surmount both  $B+C$  versus  $C$  alone

As noted above, Schild plots for the test antagonist (figure 2-4A) alone and for the test antagonist plus a range of reference antagonist (figure 2-4B) are obtained. Equieffective dose ratios are compared. A term  $\phi$  is defined as the ratio of reference antagonist concentrations giving equal dose ratio-1 values in the presence of various concentrations of test antagonist:

$$\log(\phi - 1) = \log[C] - \log K_{D_C} \quad (2.28)$$

and a replot, as in figure 2-4D, is obtained. Thus, if the test antagonist,  $C$ , is a simple competitive antagonist, a plot of  $\log(\phi - 1)$  versus  $\log[C]$  will yield a straight line, with a slope of 1. If the linear regressions can be fit to a common slope of 1.0 (i.e. if 1.0 is within the 95% confidence limits of each of the slopes), then a refit of the data in figure 2-4D to a slope of unity yields the  $K_{D_C}$  for the reference antagonist,  $C$ . Deviation from unity or a curvilinear relationship between  $\log(\phi - 1)$  and  $\log[C]$  is evidence of non-orthosteric antagonistic effects. As shown in figure 2-4G versus 2-4H, the resultant plot (2-4H) allows discrimination of gallamine as an allosteric inhibitor of carachol actions at the muscarinic receptors.

If the two agents both are null, or simple, competitive antagonists, then the reduction in response to agonist by these two antagonists is a function of a



factor equal to the additive concentration of antagonists, expressed as a fraction of their  $K_{D_B}$  values:

$$\frac{[B_1]}{K_{D_{B_1}}} + \frac{[B_2]}{K_{D_{B_2}}} \text{ (i.e. additive dose ratios)} \quad (2.29)$$

If the two antagonists are both null antagonists, the effect of the two antagonists on response will be additive and can be calculated by the Gaddum equation (2.16). However, if the two antagonists are not interacting at the same site on the receptor, then additive dose ratios likely will not be observed.

## SUMMARY

The methods summarized in this chapter provide approaches for characterizing the specificity of a drug or hormone effect. This specificity provides evidence for a receptor-mediated response. Once a receptor is implicated in a response, a variety of analyses are available for estimating the equilibrium dissociation constants for agonists, partial agonists, antagonists and inverse agonists. It is clear that evaluation of receptor-mediated response provides a great deal of information, both qualitatively and quantitatively, regarding ligand-receptor interactions and subsequent receptor-activated responses. These approaches were developed when the biological preparation under study was intact tissue or native target cells. However, in current high throughput screening technologies where the read-out is a cell-based response, these strategies have comparable value in elucidating the mechanism and providing quantitative parameters to describe the properties of novel agents. In fact, the only receptor parameter that cannot be obtained from studies of dose-response relationships in intact cell or tissue preparations is receptor density. Radioligand binding methods for characterization of receptors yielding information concerning receptor specificity, affinity *and* density are discussed in chapter 3.

## REFERENCES

**General**

- Ahlquist, R.P. (1948) A study of the adrenotropic receptors. *Am. J. Physiol.* 155: 586-600.
- Arunlakshana, O. and Schild, H.O. (1959) Some quantitative uses of drug antagonists. *Brit. J. Pharm.* 14:48-58.
- Barlow, R., Scott, N.C. and Stephenson, R.P. (1967) *Brit. J. Pharmacol.* 31:188-196.
- Colquhoun, D. (1973) The relation between classical and cooperative models for drug action. In *Drug Receptors*, H.P. Rang (ed.), pp. 149-182. Baltimore: University Park Press.
- Black, J.W., Gershkowitz, V.P., Leff, P. and Shankley, N.P. (1986) Analysis of competitive antagonism when this property occurs as part of a pharmacological resultant. *Brit. J. Pharm.* 89:547-555.
- DeLean, A., Stadel, J.M. and Lefkowitz, R.J. (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled  $\beta$ -adrenergic receptor. *J. Biol. Chem.* 255:7108-7117.
- Furchgott, R.F. (1972) The classification of adrenoceptors (adrenergic receptors): An evaluation from the standpoint of receptor theory. In *Handbuch der Experimentellen Pharmacologie*, vol. 33, *Catecholamines*, H. Blashko and E. Muscholl (eds.), pp. 283-335. Berlin: Springer-Verlag.
- Furchgott, R.F. (1978) Pharmacological characterization of receptors: Its relation to radioligand-binding studies. *Fed. Proc.* 37:115-120.
- Gaddum, J.H. (1937) The quantitative effects of antagonist drugs. *J. Physiol. (London)* 89:79-9P.
- Gaddum, J.H. (1943) Biological aspects: The antagonism of drugs. *Trans. Faraday Soc.* 39:323-333.
- Gaddum, J.H. (1957) Theories of drug antagonism. *Pharm. Rev.* 9:211-217.
- Kenakin, T.P. (1984a) The classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.* 36:165-222.
- Kenakin, T.P. (1987a) *The Pharmacological Analysis of Drug Receptor Interaction*. NY: Raven Press.
- Kenakin, T.P. (1987b) What can we learn from models of complex drug antagonism in classifying hormone receptors? In *Perspectives on Receptor Classification, Receptor Biochemistry and Methodology*, vol. 6, pp. 167-185. Black, J.W., Jenkinson, D.H. and Gershkowitz, V.P. (eds.). NY: Alan R. Liss.
- Kenakin, T.P. (2001) Quantitation in Receptor Pharmacology. *Rec. & Chan.* 7:371-385.
- Kenakin, T.P. (2004) *A Pharmacology Primer: Theory, Methods, and Application*. San Diego: Elsevier Press, Inc.
- MacKay, D. (1966) A general analysis of the receptor-drug interaction. *Brit. J. Pharmacol.* 26:9-16.
- MacKay, D. (1978) How should values of  $pA_2$  and affinity constants for pharmacological competitive antagonists be evaluated? *J. Pharm. Pharmac.* 30:312-313.
- MacKay, D. (1982) Dose-response curves and mechanisms of drug action. *Trends in Pharm. Sci.* 2:496-499.
- Parker, R.B. and Waud, D.R. (1971) Pharmacological estimation of drug-receptor dissociation constants: Statistical evaluation. I. Agonists. *J. Pharm. Exp. Ther.* 177:1-12.
- Schild, H.O. (1947)  $pA$ , a new scale for the measurement of drug antagonism. *Brit. J. Pharm.* 2:189-206.
- Schild, H.O. (1957) Drug antagonism and  $pA_x$ . *Pharm. Rev.* 9:242-245.

- Schild, H.O. (1973) Receptor classification with special reference to  $\beta$ -adrenergic receptors. In *Drug Receptors*, pp. 29-36, H.P. Rang (ed.). Baltimore: University Park Press.
- Stephenson, R.P. (1956) A modification of receptor theory. *Brit. J. Pharm.* 11: 379-393.
- Thron, C.D. (1973) On the analysis of pharmacological experiments in terms of an allosteric receptor model. *Mol. Pharmacol* 9:1-9.
- Van Rossum, J.M. (1963) Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.* 143:299-330.
- Waud, D.R. (1969) On the measurement of the affinity of partial agonists for receptors. *J. Pharm. Exp. Ther.* 170:117-122.

## Specific

- Berg K.A., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P. and Clarke, W.P. (1998) Effector Pathway-Dependent Relative Efficacy at Serotonin Type 2A and 2C Receptors: Evidence for Agonist-Directed Trafficking of Receptor Stimulus. *Mol. Pharm.* 54:94-104.
- Berthelson, S. and Pettinger, W.A. (1977) A functional basis for classification of  $\alpha$ -adrenergic receptors. *Life Sci.* 21:595-606.
- del Castillo, J. and Katz, B. (1957) Interaction at endplate receptors between different choline derivatives. *Proc. Royal Soc. Lond., Series B*, 146:369-381.
- Furchgott, R.F. (1954) Dibenzamine blockade in strips of rabbit aorta and its use in differentiating receptors. *J. Pharm. Exp. Ther.* 111:265-284.
- Furchgott, R.F. (1966) The use of  $\beta$ -haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. In *Advances in Drug Research*, vol. 3, pp. 21-55. Harper, N.J. and Simmonds, A.B. (eds.). New York: Academic Press.
- Furchgott, R.F. and Bursztyn, P. (1967) Comparison of dissociation constants and relative efficacies of selective agonists acting on parasympathetic receptors. *Ann. NY Acad. Sci.* 139:882-889.
- Kaumann, A.J. and Marano, M. (1982) On equilibrium dissociation constants for complexes of drug receptor subtypes: selective and nonselective interactions of partial agonists with two  $\beta$ -adrenoceptor subtypes mediating positive chronotropic effects of (-) isoprenaline in kitten atria. *Naunyn Schmiedeberg's Arch. Pharmacol.* 219:216-221.
- Kenakin, T.P. (1984b) The relative contribution of affinity and efficacy to agonist activity: organ selectivity of noradrenaline and oxymetazoline with reference to the classification of drug receptors. *Br. J. Pharmacol.* 81(1):131-141.
- Kenakin, T.P. and Boselli, C. (1989) Pharmacologic discrimination between receptor heterogeneity and allosteric interaction: resultant analysis of gallamine and pirenzepine antagonism of muscarinic responses in rat trachea. *J. Pharmacol. Exp. Ther.* 250(3):944-952.
- Kono, T. and Barham, F.W. (1971) The relationship between the insulin-binding capacity of fat cells and the cellular response to insulin. *J. Biol. Chem.* 246: 6210-6216.
- Kuwasako, K., Cao Y-N., Nagoshi, Y., Tsuruda, T., Kitamura, K. and Eto, T. (2004) Characterization of the Human Calcitonin Gene-Related Peptide Receptor Subtypes Associated with Receptor Activity-Modifying Proteins. *Mol. Pharm.* 65:207-213.
- Langer, S.Z. and Trendelenberg, U. (1960) The effect of a saturable uptake mechanism on the slope of dose-response curves for sympathomimetic amines and on the shifts of dose-response curves produced by a competitive antagonist. *J. Pharm. Exp. Ther.* 167:117-142.
- Leff, P., Dougall, I.G., Harper, D.H. and Dainty, I.A. (1990) Errors in agonist affinity estimation: Do they and should they occur in isolated tissue experiments? *Trends in Pharm. Sci.* 11:64-67.

- Lefkowitz, R.J., Cotecchia, S., Samama, P. and Costa, T. (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends in Pharm. Sci.* 14:303-307.
- Lin, C.W. and Musacchio, J.M. (1983) The determination of dissociation constants for substance P and substance P analogues in the guinea pig ileum by pharmacological procedures. *Mol. Pharmacol.* 23:558-562.
- Paton, W.D.M. and Rang, H.P (1965) The uptake of atropine and related drugs by intestinal smooth muscle of the guinea pig in relation to acetylcholine receptors. *Proc. R. Soc. Lond. B.* 163:1-44.
- Schultz, R., Wuster, M., Krenss, H. and Hers, A. (1980) Lack of cross-tolerance on multiple opiate receptors in the vas deferens. *Mol. Pharmacol.* 18:395-401.
- Schütz, W. and Freissmuth, W.B. (1992) Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends in Pharm. Sci.* 13:376-380.
- Sine, S.M. and Taylor, P. (1981) Relationship between reversible antagonist occupancy and the functional capacity of the acetylcholine receptor. *J. Biol. Chem.* 256:6692-6699.
- Tallarida, R.J., Cowan, A. and Adler, M.W. (1979)  $pA_2$  and receptor differentiation: A statistical analysis of competitive antagonism (minireview). *Life Sci.* 25:637-654.

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