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COMMENTARY

Qualitative and Quantitative Assessment of Relative Agonist Efficacy

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ABSTRACT. Historically, the ability of a ligand to bind to its receptor and the ability to subsequently activate that receptor have been described as the properties of affinity and intrinsic efficacy, respectively. These properties were originally believed to be independent of one another; both are possessed by ligands classed as "agonists," and they have served as the quantitative foundation of the drug and receptor classification process. Although affinity has been interpreted readily in physicochemical terms, equivalent molecular models for efficacy remain elusive. In recent times, there has been a significant paradigm shift in our understanding of the interrelationship between affinity and intrinsic efficacy, particularly on theoretical grounds, yet the actual methods available to measure these parameters remain largely operational. Nevertheless, a number of approaches, based on both functional measurements and radioligand binding studies, are available to quantify agonist efficacy on a relative scale and, to date, these remain the most practical. This commentary discusses the most common of these methods, their advantages and limitations, the dependence of the expression of agonism on the chosen assay system, and the impact of recent biochemical and molecular biological advances on the study of efficacy. Additionally, some of the more contemporary theories regarding the molecular nature of efficacy are briefly discussed, as well as the caveats that always must be borne in mind when any determinations of relative agonist efficacy are made. BIOCHEM PHARMACOL **58**;5:735–748, 1999. © 1999 Elsevier Science Inc.

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By definition, ligands classed as "agonists" possess the dual properties of ***affinity*** and ***intrinsic efficacy*** with regard to their interaction with receptors, whereas "antagonists" possess only affinity [1]. The property of affinity describes the avidity or the tenacity with which a ligand will bind to its receptor. It can be described in molecular terms and is physicochemical in nature; namely, it is the reciprocal of the microscopic equilibrium dissociation constant (K_A) for the ligand–receptor interaction. Historically, intrinsic efficacy has been viewed as the property of a ligand that enables it to produce a biological response [1]. This definition, however, inextricably links the expression of "efficacy," and hence the expression of "agonism," to the existence of a suitable assay system that will allow a change in biological state to be measured. As a consequence, a definitive molecular characterization of the property of intrinsic efficacy in a manner that transcends the biological test system while remaining applicable to a specific ligand–receptor pair has remained elusive [2]. Without a suspected molecular mechanism of intrinsic efficacy, therefore, any measurements of this latter property by necessity have been made on an operational level.

To date, the most commonly applied methods for estimating agonist affinity and relative intrinsic efficacy have relied on a number of assumptions (Table 1). With the increased sensitivity afforded by more recent biochemical approaches to the quantification of agonism, as well as the explosive advances in the field of molecular biology, a number of concerns have been raised about the theoretical framework in which affinity- and efficacy-related parameters traditionally have been estimated. Nevertheless, the classic approaches to the measurement of relative intrinsic efficacy, although strictly operational, are still the most readily applied and widely used. As such, this commentary will provide a brief overview of the most common approaches to the measurement of relative agonist efficacy, their strengths and limitations, and some newer alternatives.

DEVELOPMENT OF THE CONCEPT

The earliest application of mathematical principles to the measurement of drug action at receptors is credited to A. J. Clark [3] and has been termed "occupation theory." In essence, occupation theory is based on mass-action kinetics and attempts to link the action of a drug to the proportion

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TABLE 1. Traditional assumptions of occupation theory*

1. The interaction between a ligand and a receptor is bimolecular and readily reversible, following the law of mass action.
2. The response results from equilibrium (or steady-state) occupation of the receptors.
3. The response results from the stimulus, which is proportional to the concentration of agonist–receptor complexes formed.
4. A maximal stimulus occurs when all receptors are occupied.
5. For any given tissue, the stimulus–response relationship is characteristic of that tissue.
6. A negligible amount of drug is taken up by the receptors or the tissue, relative to the concentration of drug added.
7. Irreversible alkylation of receptors does not modify the stimulus–response relationship.

*Adapted from Ref. [1].

of receptors occupied by that drug at equilibrium. It was not until the seminal work of Ariëns [4] and Stephenson [5], however, that an explicit attempt was made to separate the binding phenomenon from the activation phenomenon within the theoretical framework of occupation theory. This work was based on a significant amount of structure–activity studies that appeared to indicate that affinity and intrinsic efficacy were independent of one another. These studies also led to the discovery of agonists that were not capable of producing a maximal tissue response, even at saturating concentrations. Ariëns termed these drugs ***dualists***, whereas Stephenson called them ***partial agonists***. Agonists that did appear to produce the maximal tissue response were, accordingly, redefined as ***full agonists***. Stephenson went further and removed the assumption that maximal receptor occupancy was a prerequisite for maximal response, which was implicit in the theoretical treatments of both Clark and Ariëns. In doing so, Stephenson introduced both the concepts of "stimulus" and "efficacy." Hence, a broad classification of drugs based on their efficacy (full agonist, partial agonist, antagonist) became possible, but the danger of this approach was, and always will be, in its phenomenological nature, i.e. the dependence of both affinity and efficacy on the physiological system in which the response is being measured (see below) does not allow these classifications to apply beyond the particular assay system in which they are assigned. Furchgott [6] recognized that an important component of the system dependence of efficacy could be attributed to differences in the amount of receptors available from tissue to tissue, and thus defined "intrinsic efficacy" as the property of a drug capable of imparting a stimulus *per receptor molecule*. However, the system dependence of agonism relies on other factors as well (see below).

Interestingly, at around the same time that Ariëns and Stephenson first explicitly addressed the issue of efficacy, Katz and Thesleff [7] applied the first "mechanistic" model to the study of drug–receptor interaction. While studying the ligand-induced desensitization observed at the nicotinic acetylcholine receptor in motor end plates, they postulated that the ligand-bound receptor is able to undergo a ***conformational change*** to a desensitized ***state***. An extended version of their model even postulated that the receptor underwent such a change in the absence of ligand. This type of allosteric transition, or ***isomerization***, thus allows receptors to exist in multiple states. Receptors are proteins, and it is now well established that proteins possess unique thermodynamic properties that allow them to adopt a variety of conformations, even in the absence of other interacting molecules [8]. The isomerization mechanism of Katz and Thesleff thus would prove to be one of the foundations of the more contemporary concepts in receptor theory. Another important concept, arising directly from a consideration of the proteinaceous nature of receptors, was that of their ability to translocate within the membrane and to interact with other proteins [9]. These concepts have culminated today in a number of variants of the "ternary complex model" [10–13] for G protein-coupled receptors. For the ion-channel linked receptors, the isomerization scheme of Katz and Thesleff has been extended to include even more conformational states [14].

The cell may be viewed as a societal realm of receptors, transducers, and effectors, possessing their own intrinsic behavior in the absence of ligand [15]. The introduction of a ligand, whether an endogenous hormone or an exogenous drug, into such a system, therefore, may result in an alteration of the state of the system such that the subsequent interaction of a receptor with other membrane components is modified. This change in basal state may be toward an increased probability of signaling ("positive efficacy") or a decreased probability of signaling ("negative efficacy"). Zero efficacy would result when the ligand-associated receptor resides in a tertiary conformation that is equivalent, with respect to basal signaling, to that found in the absence of ligand. In a nutshell, therefore, the contemporary view of "agonism" requires that the drug-bound receptor exists in an ***isomerized*** state such that its subsequent interactive properties are altered with respect to those of the unbound state [16]. A consequence of this idea has been the realization that a "spectrum" of efficacy, ranging from positive to negative, can exist and that two more phenomenological descriptors may be applied to drugs, based on their efficacy, i.e. ***inverse agonists***, those compounds that display negative efficacy, and ***neutral antagonists***, those compounds with truly zero efficacy.

Unless a ligand possesses zero efficacy, the simplest extension of the mass-action models would require that the receptor be distributed among at least three states, denoted here as R_{free}, R_{bound}, and R^*_{bound}, with the latter state denoting the bound and activated complex. In this type of schematic, the "R*" species may be viewed as representing an isomerized state of the receptor, the magnitude of which is proportional to the intrinsic efficacy of the ligand. For ion channel-linked receptors, this species may represent the agonist-bound open channel state. For G protein-coupled receptors, this may be the form of the receptor that binds to and activates the G protein. Figure 1 shows a general

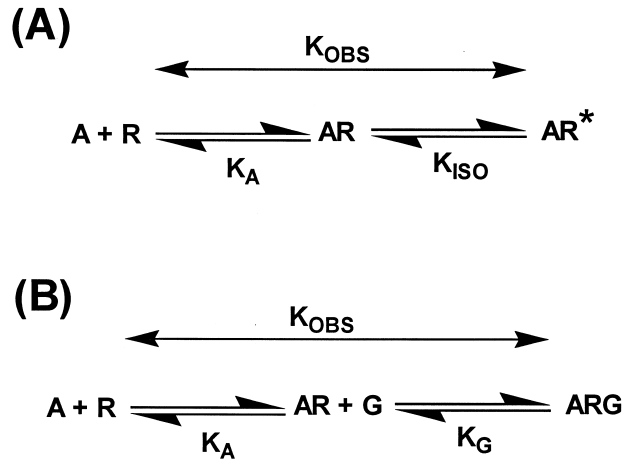

FIG. 1. Agonist-promoted receptor isomerization. (A) Binding of agonist A to the free receptor R is governed by the microscopic equilibrium dissociation constant K_A and promotes the isomerization of the AR species to AR* according to the microscopic isomerization constant, K_{ISO}. (B) As for (A), but the isomerization process yields an AR species that is capable of recognizing G protein and binding it according to the microscopic equilibrium dissociation constant, K_G. In each case, the observed macroscopic agonist dissociation constant (K_{OBS}) is influenced by both microscopic equilibrium constants.

schematic diagram of a simple isomerization mechanism, as well as a specific example of such a mechanism when applied to the interaction between an agonist, a receptor, and a G protein. The existence of basal, agonist-independent activity [17] introduces yet a fourth state, R^*_{free}. With the advent of molecular biological techniques and their application to the pharmacological arena, the actual molecular nature of intrinsic efficacy has begun to be investigated. These types of studies afford the pharmacologist an opportunity to probe the possible conformations involved in the formation of active ligand–receptor complexes (see below).

THE SYSTEM DEPENDENCE OF AGONISM

The mathematical expression of the concepts embodied in classic occupation theory is presented below in Equation 1:

$$\frac{E_A}{E_M} = f(S) = f\left(\frac{\epsilon_A \cdot [R_T] \cdot [A]}{[A] + K_A}\right) \quad (1)$$

This relationship provides the basis for the majority of methods utilized to quantify affinity and relative efficacy. It states that the magnitude of the observed effect (E_A), for any given concentration of ligand A, relative to the maximal possible tissue effect mediated by the receptor and its associated transduction pathways (E_M), is a monotonic and continuous function (f) of the "stimulus," or signal (S), produced by the drug–receptor interaction. Of the four parameters in the equation, two represent unique properties of the ligand itself (intrinsic efficacy, ϵ_A, and the equilibrium dissociation constant, K_A). It is well known that K_A is expressed in concentration units. However, it is often overlooked that ϵ_A also possesses units, namely, the reciprocal of receptor concentration [6]. The product of ϵ_A and $[R_T]$ thus yields the dimensionless parameter, e, that Stephenson [5] originally defined as "efficacy" in his formulation of receptor theory. The other two parameters in Equation 1, however, are ***tissue- or system-dependent***, i.e. receptor density (R_T) and the function, f, denoting the efficiency of stimulus–response coupling [2, 5, 6, 18]. From Equation 1, it may be seen that the initial signal imparted by a drug to a cell will be translated into an observable response depending on the number of available signaling units (i.e. receptors) and the efficiency with which the post-receptor cellular machinery (enzymes, effectors, etc.) can process the signal. These latter factors can vary from tissue to tissue. Thus, the response to any drug with, theoretically, the *same* affinity and intrinsic efficacy for a particular receptor *still may vary* depending on the chosen biological assay system. This is better appreciated when one realizes that there is generally a *nonlinear* relationship between stimulus and response, with the (rare) exception of very weak partial agonists [1]. One of the simplest nonlinear functions is the rectangular hyperbola. If response is a hyperbolic function of stimulus, then Equation 1 may be rewritten as:

$$\frac{E_A}{E_M} = \frac{S}{S + \beta} \quad (2)$$

or, by substitution into Equation 1:

$$E_A = \frac{E_M \cdot \left(\frac{e}{e + \beta}\right) \cdot [A]}{[A] + K_A \cdot \left(\frac{\beta}{e + \beta}\right)} \quad (3)$$

where e denotes the product $\epsilon_A \times [R_T]$ and β is a fitting parameter that encompasses the efficiency of post-receptor stimulus–response coupling. The smaller the value of β, the greater the efficiency of this coupling. Figure 2 illustrates a series of simulated concentration–response curves obeying this relationship, where β is the only parameter that is varied. This is equivalent to testing the same agonist at the same receptor (present at the same density) but in different tissues with varying efficiencies of post-receptor coupling. Even if comparisons were to be made within the same tissue (constant β and $[R_T]$), Equation 3 still indicates that *both* the location *and* the maximal asymptote of the resulting concentration–response curve would be influenced by the efficacy of the agonist, the location now being defined by $K_A(\beta/e + \beta)$ and the maximal asymptote as $E_M(e/e + \beta)$. The larger the value of e, or the smaller the value of β, the closer the concentration–response curve asymptote will approach the tissue maximum, and the greater the leftward shift of this curve would be from the concentration–occupancy curve (dotted curve in Fig. 2). The finding of a

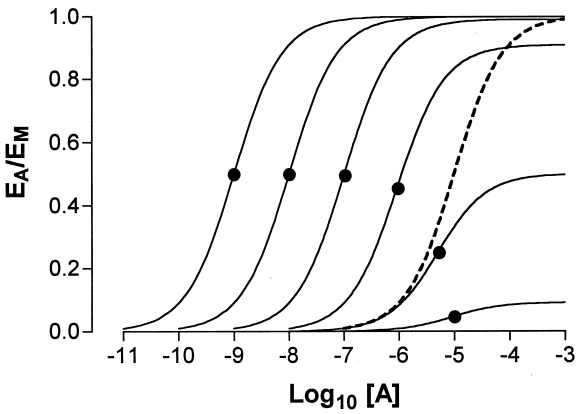

FIG. 2. System dependence of agonism. Simulated agonist concentration–response curves (solid lines) for the same agonist, acting at the same receptor, under conditions of varying efficiency of stimulus–response coupling as would be observed, for example, in different tissues. Simulations were according to Equation 3 in the text with the following parameters: K_A = 10 μM, e = 10, E_M = 1. For each individual curve (left to right), the parameter β was 0.001, 0.01, 0.1, 1, 10, and 100, simulating successive decrements in efficiency of stimulus–response coupling. Solid circles denote the midpoint location of each concentration–response curve. The dashed curve represents the concentration–occupancy relationship for the agonist.

maximal response to an agonist at submaximal receptor occupancy in a particular system identifies that system as possessing a "receptor reserve," or "spare receptors" [1, 19]. In reality, the term "receptor reserve" should not be interpreted in terms of specific "requisite" and "spare" receptor pools, but, instead, should be viewed as a synonym for "nonlinear stimulus response coupling."

For the above reasons, absolute functional measurements of the drug–receptor interaction within a particular assay system cannot be relied upon to furnish uncontaminated estimates of affinity or intrinsic efficacy. Nevertheless, a number of methods have been developed to nullify the impact of system-dependent effects on the expression of agonism in functional assays. Where this is not possible, the system dependence of agonism, as long as it is recognized, can still be exploited when comparisons are made in the same system, and this is discussed in the next section.

COMMON FUNCTIONAL APPROACHES TO MEASURING RELATIVE EFFICACY

Intrinsic Activity

Ariëns [4] originally introduced the concept of ***intrinsic activity*** (denoted by the α) as a molecular property of a ligand that is related to its efficacy, with antagonists possessing α values of 0, full agonists values of 1, and partial agonists having values anywhere in between. In light of subsequent findings, the original concept of intrinsic activity was found inadequate to describe efficacy on a molecular level. Although able to account for agonists that could not produce the maximal tissue response at receptor-saturating concentrations, intrinsic activity could not account for drugs yielding a maximal tissue response at submaximal receptor occupancies (because this requires a nonlinear stimulus–response relationship).

Nevertheless, the introduction of the concept of intrinsic activity solidified the practice of utilizing the maximal asymptote of a concentration–response curve as an index of agonist efficacy. In fact, the maximal response to an agonist, derived from a complete concentration–response curve, is probably the best, and one of the easiest, indices of efficacy available to pharmacologists. Today, the recommended usage of intrinsic activity is simply as a ratio of an agonist's maximal response to that of the maximal response of a full agonist in the same assay system [20]. The term, therefore, is entirely descriptive with no assumptions about mechanism. The limitations of this approach are obvious; intrinsic activity cannot differentiate between full agonists, and it is naïve to make the assertion that two drugs have the same "efficacy" simply because they produce the same maximal response. Additionally, intrinsic activity will always be influenced by tissue-related, as well as drug-related parameters (see Equation 3). However, ***relative*** intrinsic activity, when assessed in the same assay system, is still an easy and useful measure when comparisons are made between partial and full agonists, or amongst partial agonists, as conclusions may then be drawn about relative agonist efficacy.

More recently, Lew [21] has presented a practical means of constructing "extended" concentration–response curves that can be used to overcome the normal constraints of a tissue maximum such that a better discrimination may be made between drugs classed as "full agonists." In essence, the technique involves the use of a functional antagonist to maintain the tissue in a state whereby changes in stimulus are translated into measurable responses. For instance, if a relaxant response is being measured, and the agonist causes full tissue relaxation, then a contracting agent, acting via another signaling pathway, is added to alter tissue state such that further increments in relaxant agonist concentrations may be made. By utilizing the ***change*** of the measured tissue state parameter from its value immediately prior to an agonist concentration increment as the "response," and cumulating these responses, an extended concentration–response curve may be constructed that bypasses the conventional "tissue maximum." This approach, therefore, is a useful extension of the intrinsic activity scale of Ariëns.

Another more common, yet unreliable, approach that is often utilized in the comparison of relative agonist efficacy is one where responses to single, "saturating," concentrations of different agonists are compared. Although often thought of as a simplified method of comparison of intrinsic activities, this procedure is fraught with a number of potential errors. For instance, the system-dependence of agonism means that a concentration of agonist that may appear to be "saturating" with regard to response generation in one assay cannot be assumed to be so in another type of assay. Even more insidious are the effects that additional processes, such as desensitization, actions at two-receptor

systems, and secondary (non-receptor) drug actions, can have on the concentration–response relationship, often yielding "bell-shaped" concentration–response curves, such that higher concentrations of agonist may appear less efficacious than lower concentrations [22–24]. If any of these types of processes are occurring in the assay system under study, then comparison of supposedly "maximal" agonist effects is an invalid approach to quantifying relative efficacy.

Relative Intrinsic Efficacy

The methods outlined so far are limited in that they are almost invariably "colored" by the properties of the assay system in which responses are being measured. Null methods have been developed, however, that aim to furnish relative estimates of agonist intrinsic efficacy. Practically, the methods that are most commonly utilized in measuring relative intrinsic efficacy require the prior determination of the affinity of, at least, one of the agonists being compared [25]. Although Mackay [26] has presented a method for determining rank order of efficacy for two agonists that does not require prior knowledge of agonist affinity, this approach is limited, especially when full agonists are compared, and is not often used.

By far, the most common approach to the estimation of relative agonist intrinsic efficacy has been the "*receptor inactivation method*" introduced by Furchgott and colleagues [6, 27]. In this method, agonist concentration–response curves are constructed before and after irreversible alkylation of a significant fraction of the total receptor population. Equieffective agonist concentrations before ([A]) and after ([A′]) the alkylation procedure, determined at various response levels, are then compared in order to find K_A. From Equation 1, these concentrations may be shown to be related to one another according to the following equation:

$$[A] = \frac{[A'] \cdot q \cdot K_A}{[A'] \cdot (1 - q) + K_A} \tag{4}$$

where q represents the fraction of remaining, non-alkylated receptors.

Nowadays, with the ready availability of powerful computing packages, K_A and q are best estimated by fitting a plot of [A] versus [A′] to Equation 4 by nonlinear regression. In the past, a number of linear transformations of Equation 4 have been utilized, but these are considered to be statistically flawed and best avoided where possible [1].

Once K_A is established, it then may be utilized in constructing an agonist *occupancy–response curve*. When occupancy–response curves of different agonists are compared, their relative locations may be used to estimate their relative efficacies, as the greater the efficacy, the greater the expected leftward translocation of an agonist occupancy–response curve [1]. As with all methods for measuring affinity and/or efficacy, this approach is not without its own problems. First, a suitable alkylating agent, lacking nonspecific actions, must be available for the particular receptor system under study, and this is not always possible. Second, occupancy–response curves must, theoretically, be parallel such that an unambiguous estimate of translocation may be made. This means that statistical testing of parallelism will be required. Third, for low efficacy partial agonists, it may not be possible to construct a complete occupancy–response curve [1]. Nevertheless, the inactivation method remains the most widespread functional approach for calculating agonist affinity and relative efficacy.

The Operational Model of Agonism

Black and Leff [28] have presented an alternative method for analyzing concentration–response data to the traditional null methods based on occupation theory. By assuming an explicit, mathematical formulation (general logistic) of the "transducer function" linking agonist-receptor occupancy to response, and by coupling this function to the mass-action-based, rectangular-hyperbolic occupancy function, the following, explicit relation between agonist concentration, [A], and pharmacological effect, E_A, may be derived:

$$E_A = \frac{E_M \cdot \tau^n \cdot [A]^n}{(K_A + [A])^n + \tau^n \cdot [A]^n} \tag{5}$$

where K_A and E_M are as defined previously, n is the slope of the transducer function linking occupancy to response, and τ is the operational definition of efficacy. This latter parameter may be defined algebraically as $[R_T]/K_E$, where K_E represents the concentration of agonist–receptor complex yielding half the maximal effect. Because the τ parameter contains both tissue and drug-receptor-specific properties, it is not conceptually equivalent to intrinsic efficacy. As the name implies, however, the operational model was designed to make minimal assumptions about mechanism, and τ values are still readily utilized as measures of relative intrinsic efficacy when comparisons are made in the same assay system.

Because Equation 5 is an explicit description of an agonist concentration–response relationship, the operational model offers the advantage over the Furchgott method of being able to be fitted directly to non-transformed agonist concentration–response data. The only requirement is that the curves be described by a general logistic shape, as is often observed with most agonist concentration–response curves. Thus, for any one agonist, if a series of concentration–response curves are obtained in a tissue with varying $[R_T]$, as occurs in the inactivation method, then the entire family of curves may be fitted simultaneously to Equation 5 with all parameters except τ constrained to be shared. In this way, single estimates may be made of K_A, n, and E_M that describe the entire data set. Additionally, the q parameter obtained from the Furchgott inactivation method may also be obtained via application of the operational model, i.e. the ratio of τ obtained after

alkylation to that obtained before, τ'/τ, is equivalent to $(1 - q)$ in the Furchgott formulation [29].

An advantage of inactivation methods in the assessment of agonist affinity and relative efficacy is that they may be applied to both full and partial agonists. For the latter agents, additional "comparative" methods are also available, for both affinity estimation [30–32] and relative efficacy comparisons [20, 31, 33]. These methods are called "comparative" because they always relate the response of a partial agonist to that of a full agonist in a system with significant receptor reserve. If these latter two criteria are not met, then these approaches are not applicable.

RADIOLIGAND BINDING STUDIES IN THE DETERMINATION OF RELATIVE AGONIST EFFICACY

Radioligand binding studies have offered pharmacologists an alternative to the complexities of the isolated tissue, particularly in the ability to directly test the assumptions of Langmuirian kinetics as appropriate descriptors of ligand binding. To a great extent, the simple law of mass action has proven successful in describing the binding of a great number of ligands (particularly antagonists) to receptors, yet complex binding curves, demonstrating deviations from Langmuirian behavior, have been associated frequently with agonist binding [1, 34]. The simplest explanations that have been invoked were purely affinity-related, namely, that the ligand recognizes multiple, independent binding sites with different affinities [34]. However, as mentioned above, agonists are able to cause receptor isomerization in a manner that is proportional to their efficacy, and complex agonist binding isotherms may thus arise due to interconversion of the receptor protein between various isomerization states [34].

Binding experiments measure total radioligand bound to all forms of a receptor. If an isomerization process were operative, then analysis of data derived from these types of experiments would yield an observed agonist affinity that would, in fact, be influenced by the avidity of any additional agonist-receptor isomerization [1, 20, 35]. In other words, a composite K_A value ("K_{OBS}"; Fig. 1) will be measured that contains an efficacy component, as well as an affinity component. The implications of this phenomenon are significant, in that they suggest an interdependence of affinity and intrinsic efficacy to the extent that one cannot be measured independently of the other. This, of course, goes against the tenets of traditional occupation theory, and potentially invalidates the theory behind all current approaches to measure agonist affinity and intrinsic efficacy. Although most studies addressing this phenomenon have, to date, not shown significant deviations in agonist affinity estimates predicted by the theory of receptor isomerization [36], this is probably due to the fact that these experiments were functional in nature, where the presence of signal amplification meant that significant receptor isomerization was not required to observe full pharmacological effect. When agonist action is measured at the level of receptor binding, however, the impact of isomerization mechanisms on affinity and efficacy measurements is expected to be greater.

The "GTP Shift"

A practical, although not necessarily physiological, advantage of radioligand binding experiments relative to functional assays is that the conditions utilized for the binding experiment may be modified to a greater extent than in functional assays in order to optimize the detection of agonist-promoted receptor isomerization. For example, with the G protein-coupled receptors, it has been well established that the use of broken cell preparations, in low ionic strength media containing magnesium and devoid of sodium, promotes ternary complexation [37]. Under these conditions, agonist binding curves are often "shallow" (midpoint slopes less than unity) and may appear multiphasic [34]. Analysis of the data according to a multiple, independent-site model invariably resolves agonist binding into at least two apparent affinity states, often denoted K_H (high affinity) and K_L (low affinity). In many of these instances, the agonist-induced isomerization, resulting in the steady-state accumulation of high-affinity agonist–receptor–G protein ternary complex, could be effectively cancelled by the addition of GTP (or a non-hydrolyzable analogue), the so-called "GTP shift." The resultant binding curve is monophasic and reflects the lower affinity of the bimolecular agonist–receptor complex (K_A). Although it often is believed that K_L is equivalent to K_A, this is NOT the case. All affinity estimates based on a multi-site, independent binding model will be influenced, to differing extents, by an isomerization mechanism.

The ternary complex model [10] was the first mechanistic description of an allosteric interaction between an agonist and a G protein binding to the same receptor. This model predicts that the GTP-shift, i.e. the ratio of the higher agonist affinity (K_H) obtained in the absence to that obtained in the presence of GTP (K_A), may be used as a quantitative, biochemically derived measure of the efficacy of the ligand *within the particular assay system*. This latter point is important, because the observed agonist potency in these types of studies also will be determined by the stoichiometry of receptors to G proteins, and thus may vary between different systems. Hence, the appropriate practical application of the GTP shift is when comparisons are made between different ligands in the same assay systems. Under these circumstances, the GTP-shift may be used as an *ad hoc* measurement of relative efficacy [38]; the greater the shift, the greater the efficacy.

The GTP-shift, although very useful, is not infallible. The ternary complex model predicts that saturating concentrations of guanine nucleotide should result in a complete uncoupling of the receptor from the G protein, yielding a monophasic agonist binding curve that reflects agonist occupancy of the free receptor. However, there are

a number of cases where this is not observed [39], suggesting that for some systems, the ternary complex model is an inadequate mechanistic descriptor of complex agonist binding isotherms. Even if a ternary complex mechanism were operating, Waelbroeck *et al.* [40] have demonstrated how GTP-shifts may still be undetectable, or where shifts may be seen where they would not be expected (e.g. with antagonists). The reasons, in these latter instances, may be related to the kinetics of ligand-promoted exchange between nucleotides on the G proteins.

In any instance, the GTP-shift cannot be universally applied to the detection and quantification of relative agonist efficacy in radioligand binding experiments, and alternative methods need to be explored. One such approach has been presented by Kenakin [1] and termed ***"high-affinity selection."*** This method requires a recombinant expression system whereby receptors are overexpressed such that they outnumber the accessible pool of G proteins. Subsequently, agonist competition binding isotherms obtained using a radiolabeled agonist are compared with those obtained using a radiolabeled neutral antagonist. As with the GTP-shift, the ratio of agonist affinities derived from both types of competition experiments may be taken as a measure of relative efficacy. However, the same caveat applies as before, i.e. observed potency is system-dependent. Additionally, the use of high-affinity selection requires the availability of radiolabeled agonist, as well as antagonist, for the receptor system of interest, and this is often not possible.

Transducer Abstraction

If more than one agonist is present in a system with fixed amounts of receptor and transducer proteins, then the resulting agonist binding isotherms will reflect competition both for the receptor ***and*** for the formation of ternary complex. According to the full ternary complex model (Fig. 3), the abstraction of G protein by one agonist from another would be related to the magnitude of agonist efficacy. Thus, we propose that a change in the pattern of a complex agonist binding isotherm, observed in the presence of a second agonist acting via the same receptor-transducer system, can be utilized to detect agonist efficacy in radioligand binding experiments.

Figure 4 illustrates this phenomenon, using theoretical curves generated according to the full ternary complex model (Fig. 3). It may be seen that biphasic agonist/antagonist competition binding curves, constructed in the absence or presence of graded concentrations of a second drug, will demonstrate a parallel, dextral shift only if the second drug is a neutral antagonist (Fig. 4A). In contrast, if the second ligand possesses efficacy, then the curve shape also is expected to change, along with the resulting dextral shift (Fig. 4, B and C). The greater the loss of apparent high-affinity binding of the first agonist (drug A in Fig. 4, A–C), the greater the positive efficacy of the second agonist (drug C; Fig. 4B). In contrast, if the second agonist possessed negative efficacy, an apparent *increase* in the proportion of high-affinity binding state for drug A will result (Fig. 4C) that is proportional to the negative efficacy of the second agonist. Panel D of Fig. 4 illustrates these phenomena in a simple graphical format, whereby the change in the fraction of high-affinity agonist binding for drug A ($\%R_H$) is plotted against the concentration of drug C.

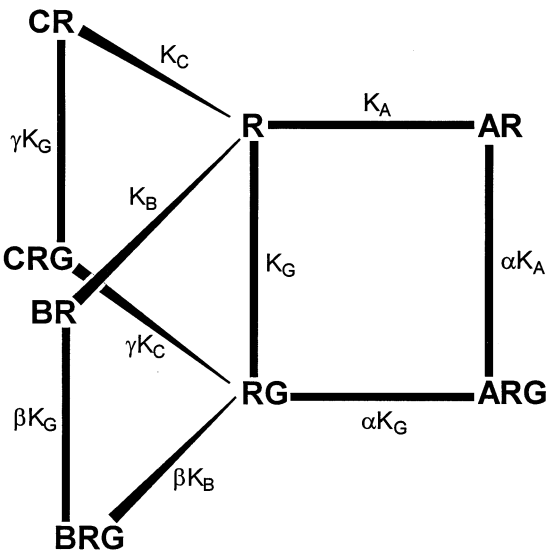

FIG. 3. Ternary complex model for three ligands, A, B and C, interacting with a common pool of receptor, R, and G protein, G. K_A, K_B, and K_C denote the ligand equilibrium dissociation constants for binding to the free receptor. K_G denotes the G protein equilibrium dissociation constant for binding to the free receptor. The dimensionless cooperativity factors, α, β, and γ, denote the magnitude by which K_A, K_B, K_C, and K_G are modified at the occupied receptor.

Figure 5 shows experimental results demonstrating the effects of the muscarinic receptor partial agonist pilocarpine on the binding of the antagonist [^{3}H]*N*-methylscopolamine (Fig. 5A), as well as on the competition between the full agonist acetylcholine and [^{3}H]*N*-methylscopolamine (Fig. 5B) at the human M_1 muscarinic acetylcholine receptor expressed in recombinant Chinese hamster ovary cells. It may be seen that competition between pilocarpine and [^{3}H]*N*-methylscopolamine did not reveal any complexity in the pattern of binding of the partial agonist. However, biphasic acetylcholine binding was readily "converted" to a monophasic curve in the presence of pilocarpine, thus reflecting the efficacy of the latter agent. Furthermore, this particular system has been shown previously to be relatively resistant to modulation by guanine nucleotides [41]. Thus, transducer abstraction can detect agonist efficacy in situations where agonist binding isotherms do not appear to indicate multiple receptor states and/or when a GTP-shift cannot be demonstrated.

Practically, the concept of transducer abstraction may be exploited in any situation where a standard full agonist, yielding a multiphasic binding isotherm, is available. Changes in the profile of this binding curve in the presence

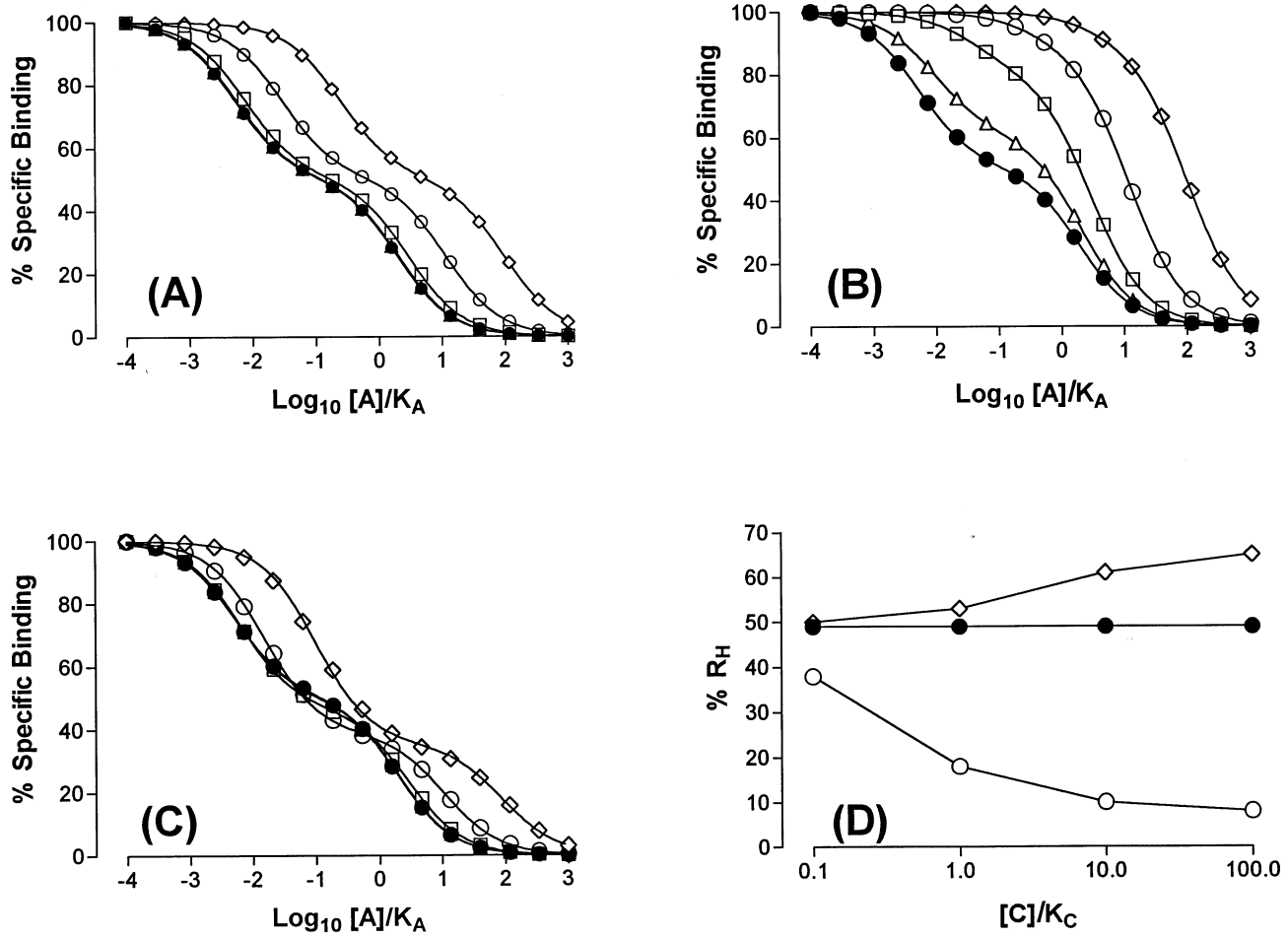

FIG. 4. Transducer abstraction, modeled according to the scheme depicted in Fig.3. Panels A–C depict the inhibition of the normalized binding of radioligand B ($\beta = 1$) by a full agonist, A ($\alpha = 0.001$), in the absence (●) or presence of graded concentrations (0.1, Δ; 1, □; 10, ○; 100, ◇; × K_C) of a second ligand, C, with the following γ values: (B) 1, (C) 0.03, and (D) 1000. For all simulations, [G]/[R] = 0.5, $K_A = K_B = K_C = K_G = 1$. Panel D depicts the change in the fraction of high-affinity binding of agonist A (%R_H) in the presence of ligand C.

of a second ligand may then be utilized to detect efficacy in the latter ligand. Importantly, the actual mechanism underlying the expression of efficacy is not necessarily important (the ternary complex model was only used as an example here) for the practical application of this approach; what matters is that the interacting drugs share this mechanism.

Combining Functional and Binding Approaches

Ehlert [42] has presented an elegant approach to estimating relative agonist efficacy utilizing a combination of both functional and radioligand binding assays. The method requires the determination of concentration–response ***and*** concentration–occupancy curves for a particular agonist, the latter most commonly inferred from a competition binding assay. Subsequently, the concentration of agonist yielding half the maximal response (EC_{50}) is compared to the concentration yielding half-maximal occupancy (K_A) according to the following relationship:

$$e = 0.5 \cdot \frac{E_{maxA}}{E_{max}} \cdot \left(\frac{K_A}{EC_{50}} + 1 \right) \tag{6}$$

where E_{max} denotes the maximum possible effect (as determined by use of a full agonist), E_{maxA} denotes the maximal effect of agonist, and e is as defined previously. For partial agonists, where the maximal response is never achieved at saturating agonist concentrations, the K_A/EC_{50} ratio becomes 1, and the equation simplifies further to:

$$e = \frac{E_{maxA}}{E_{max}} \tag{7}$$

which is equivalent to the estimation of Ariëns' intrinsic activity (but only under these latter circumstances).

Because this method requires a comparison between data obtained using two different types of experiments (binding vs function), it is most reliable when care has been taken to ensure that the experiments have been conducted under similar assay conditions, where possible. In addition, an

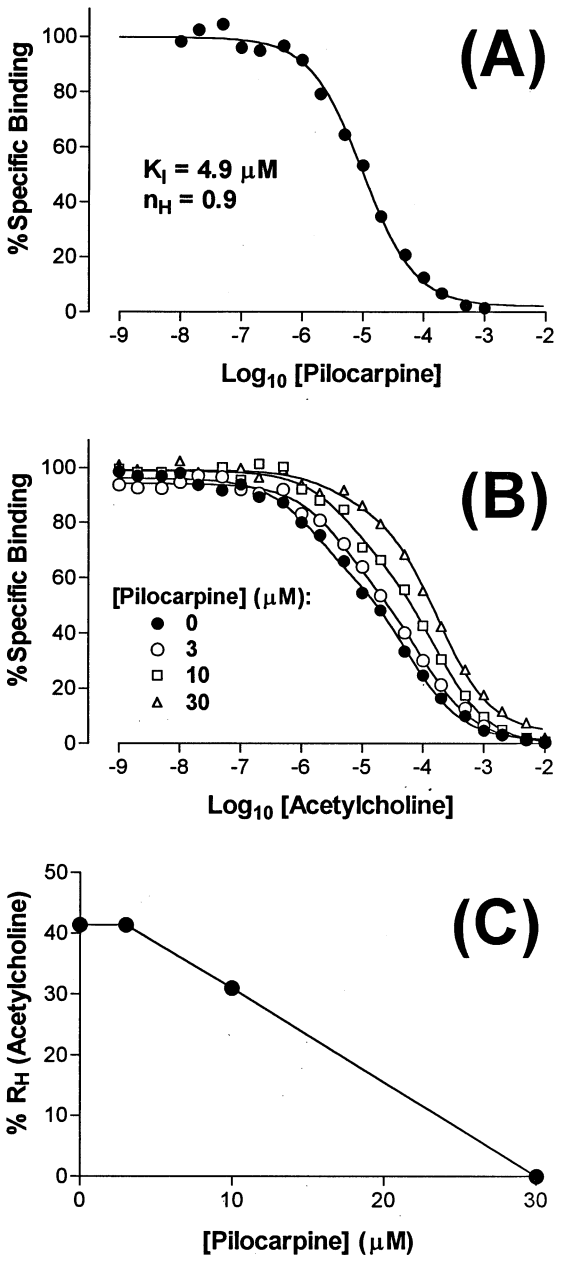

FIG. 5. (A) Inhibition of the binding of [^{3}H]*N*-methylscopolamine by pilocarpine in homogenates from Chinese hamster ovary cells expressing the human M_1 muscarinic acetylcholine receptors; K_I denotes the equilibrium dissociation constant, and n_H denotes the Hill slope factor. (B) Inhibition of the binding of [^{3}H]*N*-methylscopolamine by acetylcholine in the absence or presence of the indicated concentrations of pilocarpine. (C) Plot of the change in high-affinity acetylcholine binding in the absence or presence of pilocarpine.

unambiguous estimate of K_A is required, which often necessitates the use of broken cell preparations and inclusion of saturating GTP (or other guanine nucleotide analogs) in the binding medium, such that monophasic agonist binding isotherms are obtained (reflecting only the binary drug–receptor complex). If this is not possible, then the method cannot be employed.

IMPACT OF RECOMBINANT EXPRESSION SYSTEMS ON THE STUDY OF EFFICACY

It is now beyond question that the introduction of molecular biological techniques to the pharmacological arena has led to an explosion in our understanding of the interactive behavior between drugs, receptors, and effectors. The ability to manipulate receptor–effector stoichiometry more directly in recombinant expression systems is a useful advantage, and has allowed for the more direct testing of the dependence of affinity and efficacy on these system components [43].

In general, all the methods described in the preceding sections can be applied to the study of the drug–receptor interaction in recombinant expression systems. In addition, newer technologies have also expanded the choice of cellular "output" that researchers can exploit as measures of drug effects. For instance, signal transduction cascades more proximal to receptor activation may be monitored, if so desired. These may include agonist-mediated guanine nucleotide exchange or second-messenger accumulation, for example. Alternatively, downstream signaling events, affording greater amplification of weaker stimuli, may also be examined [43].

However, the use of recombinant technology is not without its own particular problems. Foremost amongst these is the increased probability of observing coupling of receptors to membrane or cytosolic proteins with which they do not normally interact in a native system. Since the ultimate aim of pharmacological intervention is toward the alleviation of human disease states, results obtained in recombinant systems must be shown to be useful when applied to the clinical situation. Overexpression of receptor (or G protein) can also yield aberrant or non-physiological behavior, including constitutive activity (see below) or promiscuity/pleiotropy of coupling that is not observed at low expression. With regard to the quantification of agonist efficacy, other implications of receptor overexpression also include the assignment of multiple coupling modes for receptors that do not do so in natural systems and the possible misinterpretation of results from "stop-time" assays (see Ref. 44). This latter point is of particular practical importance, as many of the functional assays performed using cell lines are not conducted in real time (unlike traditional isolated tissue experiments), but often measure cumulative product (e.g. cyclic AMP and phosphoinositides) formed over time. Similarly, radioligand binding experiments may also demonstrate a range of complex behaviors that are specific to each expression system [44].

The greatest contribution to the study of intrinsic efficacy that recombinant receptor systems have made is not so much as to define new methods for measuring relative efficacy, but rather in terms of the elucidation of the underlying molecular mechanisms of efficacy. Two general mechanisms of ligand-mediated receptor conformational change have been proposed: *conformational induction* and *conformational selection* [1, 45]. The former mechanism

implies that the binding of the ligand is a prerequisite for the creation of a tertiary conformation of the receptor protein that results in a subsequent alteration of basal signaling state. In contrast, conformational selection requires that the receptor preexists in a thermodynamic equilibrium of all possible conformational states (resting and active), and that the ligand selectively binds to one, or more, of these and shifts the preexisting equilibrium to a new one, with an accompanying change in basal signaling state. In reality, both mechanisms probably contribute to ligand–receptor conformational change to some extent. A large body of data, generated in recent times by the development of receptor mutants and chimeras, has begun to identify actual receptor domains that preferentially contribute to ligand binding or to the activation phenomenon [46–49]. Thus, the change in movement of receptor amino acid residues within the cell membrane, as a consequence of agonist binding, may be viewed as a physical manifestation of intrinsic efficacy at the molecular level. However, accurate measurements of the intramolecular environment of receptors currently are not available.

IMPACT OF CONSTITUTIVE RECEPTOR ACTIVITY ON THE STUDY OF EFFICACY

The demonstration that receptors can display basal levels of activity in the *absence* of ligand necessitated an extension of accepted models of ligand–receptor interaction [17] and has allowed for the detection and quantification of inverse agonism (negative efficacy). In general, the overexpression of wild-type receptor, whether native or recombinant, has been shown to reveal varying degrees of constitutive activity in many systems [43]. Alternatively, transducer overexpression in recombinant systems would also be expected to yield similar results [50].

Constitutively active systems also may be engineered without necessitating the overexpression of system components. The construction of receptor mutants that can readily adopt an activated state [12, 51] has been a major biological advance in terms of understanding the molecular mechanisms of efficacy. In those circumstances where these mutants show a *preferentially* higher affinity for positive agonists but an unaltered affinity for neutral antagonists, it may be assumed (tentatively) that the receptor conformation resulting from the mutation closely mimics the naturally "active" state [52]. However, there are a number of studies indicating that receptors may adopt *multiple* active states [53, 54]. For instance, the demonstration of varying levels of constitutive activity by different mutants of the same receptor may be interpreted in such a manner [51, 52]. Additionally, the construction of mutants that show constitutive activation of a specific receptor-linked transduction pathway *without* affecting other pathways linked to the same receptor is also a strong marker of multiple active states and promiscuity in receptor–effector coupling [55].

Findings such as those described above have significant implications for the quantification of relative agonist efficacy. Even if the same assay system is utilized, the system-dependent components of intrinsic efficacy may not cancel out if the comparison is between agonists that induce, or select, *different* active states of the receptor. Under these circumstances, the phenomenon of selective agonist "trafficking" of signal [53, 56] in the generation of a common final response makes unambiguous measures of relative efficacy impossible. Furthermore, the possibility that agonists may display positive efficacy in one type of system but negative efficacy in another ("protean agonists"; [57]) must be taken as a strong indicator of multiple active receptor states, the latter only becoming overtly manifested as receptor expression level is increased.

MEASURING NEGATIVE EFFICACY

The notion that intrinsic efficacy spans a continuum, ranging from positive to negative, quite readily accommodates the existence of inverse agonists. Consistent with this concept, it may be hypothesized that procedures designed to evaluate ligands with positive efficacy will also have an impact on the expression of negative efficacy, but in a converse fashion. The greatest constraint on these latter types of studies has been determined by nature itself, i.e. some degree of constitutive receptor activity is required for the detection of inverse agonists. Apart from the overexpression of receptors/transducers or the construction of receptor mutants, another established method for promoting receptor–G protein precoupling is to conduct experiments in a low-ionic strength medium, devoid of sodium ions [58, 59]. Otherwise, in a quiescent system, inverse agonist ligands will appear to behave as neutral antagonists [15].

In radioligand binding studies, the GTP-shift has also been utilized for the detection of negative efficacy. However, in these latter instances, it is better referred to as a "reverse GTP-shift," since the cancellation of receptor–G protein precoupling by guanine nucleotides would be expected to result in a ***higher*** affinity state for an inverse agonist [60, 61]. A similar effect also is expected if the G protein can be rendered effectively inaccessible to the receptor, e.g. via use of a toxin [58].

OTHER APPROACHES TO THE DETECTION AND QUANTIFICATION OF RELATIVE EFFICACY

Fluorescence Spectroscopy

One of the more recent approaches to the study of efficacy has been the use of fluorescently labeled receptors. By covalently attaching a fluorescent probe to the receptor, any ligand-induced conformational change may be detected as a spectroscopic alteration in fluorescence [62]. Binding of a ligand results in a change in the polarity of the environment surrounding the labeled portions of the receptor. With respect to the detection and quantification of efficacy, decreases in fluorescence due to agonist binding at the β_2-adrenoceptor have been observed that may be directly

correlated with the ability of the agonists employed to stimulate adenyl cyclase activity [62]. Significantly, inverse agonist binding has been associated with an *increase* in fluorescence, supporting the notion that these compounds preferentially stabilize an inactive state of the receptor [62, 63].

The use of fluorescence spectroscopy also may be extended to those systems displaying constitutive receptor activity. For example, the comparison of the fluorescence of constitutively active β_2-adrenoceptor mutants and wild-type receptors has revealed that the former display a structural instability and an exaggerated response to ligand binding, irrespective of whether the ligand possesses positive or negative efficacy [63]. These types of studies may be interpreted in terms of thermodynamic transitions between various receptor energy states and afford an unprecedented approach to the dissection of conformational changes associated with receptor binding and activation.

Receptor Desensitization

The phenomenon of an attenuated response during, or subsequent to, the initial action of a drug has long been termed "desensitization" and represents one of the primary mechanisms of homeostatic control in cellular systems [64]. It seems almost intuitive that the phenomenon should in some way be linked to agonist efficacy, yet there is a relative paucity of studies that have attempted to address this relationship in a quantitative fashion. This is most likely due to the fact that the term itself has become an "umbrella" for a number of events that are best separated on a temporal basis. In particular, the rapid uncoupling of the receptor from other response-generating elements of the cell that characterizes acute desensitization often is followed by sequestration of the receptor protein away from the cell surface and, in the face of persistent agonist presence, down-regulation of receptor protein [64]. The multitudes of intracellular signaling events that mediate these various components of signal attenuation are still being unraveled. However, some events mediating acute desensitization have been sufficiently characterized such that their relationship to agonist efficacy may be inspected.

The simplest approaches have utilized incubations of cells or tissues with equieffective concentrations of agonists of varying efficacies for a set period of time, to induce rapid desensitization, before the removal of agent and the subsequent testing of a receptor-mediated response (e.g. intracellular messenger accumulation) to a given agonist. The relative differences between maximal steady-state responses under the various conditions employed may be compared to the ability of each agonist to individually generate the same response. Correlations between intrinsic activity or coupling efficiency and ability to induce desensitization have thus been noted [65–68]. Protein reconstitution studies and associated peptide-mapping techniques have also been employed in demonstrating that the propensity of agonists to activate a particular intracellular response may be correlated with their subsequent ability to promote receptor phosphorylation by specific kinases [69].

The advent of newer techniques has also allowed for the assessment of the efficacy-dependence of longer-term desensitization events. For example, the technique of stop-flow cytometry enables real-time measurements to be made of cell-surface receptor number in the continued presence of agonist, in contrast to earlier studies that necessitated the washout of agonist prior to subsequent testing of the preparation. Utilizing such an approach, a link has been demonstrated recently between agonist efficacy and the ability to induce receptor sequestration [70]. Similar findings have been made by applying more conventional approaches to the study of agonist-mediated receptor internalization [71], and these may also be extended to incorporate agonist-mediated receptor down-regulation [72], the latter representing a useful method that does not require functional measurements.

Desensitization is not only a phenomenon associated with positive efficacy. Constitutively active mutant receptors have been shown to be constitutively desensitized and phosphorylated [73], and inverse agonists have been utilized in *reducing* the extent of this phenomenon [74]. Constitutively active wild-type receptors also have been identified that undergo basal phosphorylation and desensitization [75]. Furthermore, positive agonist-induced desensitization has been shown to modulate inverse agonist efficacy in a manner that is inversely proportional to the intrinsic activity of the latter [76].

Taken together, these findings highlight a significant link between receptor desensitization and agonist efficacy, and identify the desensitization phenomenon as a viable experimental technique for the assessment of relative agonist efficacy.

PRACTICAL SIGNIFICANCE

The aim of pharmacological intervention in the alleviation of disease states requires the ability to predict agonist activity in humans from information gleaned using *in vitro* and *in vivo* experimentation. The inability to divorce the influence of the assay system from the expression of agonism means that the current, operational measures of relative intrinsic efficacy are still the most useful means of achieving this end. For example, it is common to see the term "functionally selective" in reference to agonists that appear to exert a type of tissue selectivity by virtue of their ability to express agonism only in well-coupled receptor–effector systems. In less efficient systems, these compounds may appear indistinguishable from neutral antagonists, and this needs to be taken into account when designing or screening pharmacological agents for use in humans. The common practice of screening such ligands in cell lines expressing high receptor levels increases the probability of ascribing a degree of efficacy to these compounds that may not be retained *in vivo*, where the expression level of the target receptor is much lower. Under these circumstances, a

partial agonist detected in a cell line may behave as an antagonist *in vivo*, which would clearly be detrimental to the patient. By measuring relative agonist intrinsic efficacy, the impact of variability in stimulus–response coupling between cell lines, tissues, and species can be accounted for.

Although newer technologies have afforded the experimenter a greater choice in biological screening assays, the danger also exists that an investigator may settle on a particular assay, or assays, for rapid screening purposes that will not give useful information with regard to the potential final utility of the pharmacological agent in the treatment of the condition for which it was designed initially. The expression of agonism will change depending on which cellular response is measured in the same assay system, due to the effects of signal amplification. Obviously, the need to choose those assays that best reflect the desired clinical endpoint is paramount. The bottom line, however, is that measurement of relative intrinsic efficacy will minimize the predictive errors that may otherwise occur if only one, or a handful, of absolute functional measures were chosen to screen for agonistic activity.

CONCLUDING REMARKS

As stated by Kenakin [25], "the definition of how an agonist activates a receptor relies upon the model one chooses for the receptor." If we are to accept that receptors, like other proteins, adopt a variety of conformations in the absence of ligand, and are differentially stabilized by ligand binding, then the "binding" and "activation" steps are inextricably linked. This is because the application of thermodynamic principles to the development of models of the drug–receptor interaction will always lead to the expression of one entity (either affinity or efficacy) affecting the other. Newer concepts, based on the probabilistic models often utilized in statistical mechanics or molecular dynamics [77], have been presented in efforts to rationalize both affinity and efficacy in terms of a single, common, numerical formulation. However, the application of these concepts to pharmacology is relatively new, and has yet to yield a robust and readily applicable means for drug and receptor classification. As such, the operational approaches, based on the methods outlined in this commentary, are still the most practical means of obtaining a relative measure of ligand efficacy, provided that the experimenter remains aware of the caveats involved in making their experimental determinations. As biomedical technology evolves to the extent that changes in the intramolecular environment of the receptor protein may be correlated systematically to the expression of drug binding (affinity) or to changes in cell/tissue activation state (efficacy), then the classic approaches may be replaced eventually with newer methods that overcome the apparent system dependence of most of the current techniques.

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References

1. Kenakin TP, *Pharmacologic Analysis of Drug–Receptor Interaction*. Lippincott-Raven, Philadelphia, 1997.
2. Clarke WP and Bond RA, The elusive nature of intrinsic efficacy. *Trends Pharmacol Sci* **19:** 270–276, 1998.
3. Clark AJ, *General Pharmacology*. Springer, Berlin, 1937.
4. Ariëns EJ, Affinity and intrinsic activity in the theory of competitive inhibition. *Arch Int Pharmacodyn Ther* **99**: 32–49, 1954.
5. Stephenson RP, A modification of receptor theory. *Br J Pharmacol* **11:** 379–393, 1956.
6. Furchgott RF, The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor–agonist complexes. *Adv Drug Res* **3:** 21–55, 1966.
7. Katz B and Thesleff S, A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J Physiol (Lond)* **138:** 63–80, 1957.
8. Frauenfelder H, Sligar SG and Wolynes PG, The energy landscapes and motions of proteins. *Science* **254:** 1598–1603, 1991.
9. Cuatrecasas P, Membrane receptors. *Annu Rev Biochem* **43:** 169–214, 1974.
10. De Lean A, Stadel JM and Lefkowitz RJ, A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255:** 7108–7117, 1980.
11. Leeb-Lundberg LMF and Mathis SA, Guanine nucleotide regulation of B2 kinin receptors. Time-dependent formation of a guanine nucleotide-sensitive receptor state from which [^{3}H]bradykinin dissociates slowly. *J Biol Chem* **265:** 9621–9627, 1990.
12. Samama P, Cotecchia S, Costa T and Lefkowitz RJ, A mutation-induced activated state of the β_2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268:** 4625–4636, 1993.
13. Weiss JM, Morgan PH, Lutz MW and Kenakin TP, The cubic ternary complex receptor-occupancy model I. Model description. *J Theor Biol* **178:** 151–167, 1996.
14. Spedding M and Dacquet C, Transduction is a major factor influencing receptor characterization. *Ann NY Acad Sci* **812:** 29–40, 1997.
15. Kenakin T, Morgan P and Lutz M, On the importance of the "antagonist assumption" to how receptors express themselves. *Biochem Pharmacol* **50:** 17–26, 1995.
16. Kenakin TP, *Molecular Pharmacology. A Short Course*. Blackwell Science, Cambridge, MA, 1997.
17. Lefkowitz RJ, Cotecchia S, Samama P and Costa T, Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:** 303–307, 1993.
18. Kenakin TP, Challenges for receptor theory as a tool for drug and drug receptor classification. *Trends Pharmacol Sci* **10:** 18–22, 1989.
19. Nickerson M, Receptor occupancy and tissue response. *Nature* **178:** 697–698, 1956.
20. Jenkinson DH, Classic approaches to the study of drug–receptor interactions. In: *Textbook of Receptor Pharmacology* (Eds. Foreman JC and Johansen T), pp. 3–62. CRC Press, Boca Raton, 1996.
21. Lew MJ, Extended concentration–response curves used to reflect full agonist efficacies and receptor occupancy–response coupling ranges. *Br J Pharmacol* **115:** 745–752, 1995.
22. Szabadi E, A model of two functionally antagonistic receptor

populations activated by the same agonist. *J Theor Biol* **69:** 101–112, 1977.
23. Rovati GE and Nicosia S, Lower efficacy: Interaction with an inhibitory receptor or partial agonism? *Trends Pharmacol Sci* **15:** 140–144, 1994.
24. Pliška V, Models to explain dose–response relationships that exhibit a downturn phase. *Trends Pharmacol Sci* **15:** 178–181, 1994.
25. Kenakin TP, The quantification of relative efficacy of agonists. *J Pharmacol Methods* **13:** 281–308, 1985.
26. Mackay D, A general analysis of the receptor–drug interaction. *Br J Pharmacol* **26:** 9–16, 1966.
27. Furchgott R and Bursztyn P, Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann NY Acad Sci* **144:** 882–899, 1967.
28. Black JW and Leff P, Operational models of pharmacological agonism. *Proc R Soc Lond B* **220:** 141–162, 1983.
29. Black JW, Leff P, Shankley NP and Wood J, An operational model of pharmacological agonism: The effect of E/[A] curve shape on agonist dissociation constant estimation. *Br J Pharmacol* **84:** 561–571, 1985.
30. Barlow RB, Scott NC and Stephenson RP, The affinity and efficacy of onium salts on the frog rectus abdominis. *Br J Pharmacol* **31:** 188–196, 1967.
31. Leff P, Prentice DJ, Giles H, Martin GR and Wood J, Estimation of agonist affinity and efficacy by direct, operational model-fitting. *J Pharmacol Methods* **23:** 225–237, 1990.
32. Waud DR, On the measurement of the affinity of partial agonists for receptors. *J Pharmacol Exp Ther* **170:** 117–122, 1969.
33. Freeman KA and Tallarida RJ, Determination of the functional dissociation constant of a partial agonist by comparison with a higher efficacy agonist. *J Pharmacol Methods* **25:** 11–18, 1991.
34. Limbird LE, *Cell Surface Receptors: A Short Course on Theory and Methods*. Kluwer Academic Publishers, Boston, 1996.
35. Colquhoun D, Affinity, efficacy, and receptor classification: Is the classic theory still useful? In: *Perspectives on Receptor Classification* (Eds. Black JW, Jenkinson DH and Gerskowitch VP), pp. 103–114. Alan R. Liss, New York, 1987.
36. Leff P, Dougall IG, Harper DH and Dainty IA, Errors in agonist affinity estimation: Do they and should they occur in isolated tissue experiments? *Trends Pharmacol Sci* **11:** 64–67, 1990.
37. Hulme EC and Birdsall NJM, Strategy and tactics in receptor-binding studies. In: *Receptor–Ligand Interactions. A Practical Approach* (Ed. Hulme EC), pp. 63–176. Oxford University Press, New York, 1992.
38. Birdsall NJM and Lazareno S, To what extent can binding studies allow the quantification of affinity and efficacy? *Ann NY Acad Sci* **812:** 41–47, 1997.
39. Chidiac P, Rethinking receptor–G protein–effector interactions. *Biochem Pharmacol* **55:** 549–556, 1998.
40. Waelbroeck M, Boufrahi L and Swillens S, Seven helix receptors are enzymes catalysing G protein activation. What is the agonist K_{act}? *J Theor Biol* **187:** 15–37, 1997.
41. Van Giersbergen PLM and Leppik R, Modulation of agonist binding by guanine nucleotides in CHO cells expressing muscarinic m1–m5 receptors. *Naunyn Schmiedebergs Arch Pharmacol* **352:** 166–172, 1995.
42. Ehlert FJ, The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol Pharmacol* **28:** 410–421, 1985.
43. Kenakin T, The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* **48:** 413–463, 1996.
44. Kenakin T, Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol Sci* **18:** 456–464, 1997.
45. Burgen ASV, Conformational changes and drug action. *Fedn Proc* **40:** 2723–2728, 1981.
46. Lameh J, Cone RI, Maeda S, Philip M, Corbani M, Nádasdi L, Ramachandran J, Smith GM and Sadée W, Structure and function of G protein coupled receptors. *Pharm Res* **7:** 1213–1221, 1990.
47. Savarese TM and Fraser CM, *In vitro* mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem J* **283:** 1–19, 1992.
48. Strader CD, Fong TM, Tota MR and Underwood D, Structure and function of G protein-coupled receptors. *Annu Rev Biochem* **63:** 101–132, 1994.
49. Scheer A and Cotecchia S, Constitutively active G protein-coupled receptors: Potential mechanisms of receptor activation. *J Recept Signal Transduct Res* **17:** 57–73, 1997.
50. Burstein ES, Spalding TA, Braüner-Osborne H and Brann MR, Constitutive activation of muscarinic receptors by the G-protein G_q. *FEBS Lett* **363:** 261–263, 1995.
51. Cotecchia S, Exum S, Caron MG and Lefkowitz RJ, Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc Natl Acad Sci USA* **87:** 2896–2900, 1990.
52. Kjelsberg MA, Cotechia S, Ostrowski J, Caron MG and Lefkowitz RJ, Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at a single site. *J Biol Chem* **267:** 1430–1433, 1992.
53. Kenakin T, Agonist-receptor efficacy. II: Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:** 232–238, 1995.
54. Kenakin T, Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol Sci* **16:** 188–192, 1995.
55. Perez DM, Hwa J, Gaivin R, Mathur M, Brown F and Graham RM, Constitutive activation of a single effector pathway: Evidence for multiple activation states of a G protein-coupled receptor. *Mol Pharmacol* **49:** 112–122, 1996.
56. Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P and Clarke WP, Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: Evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54:** 94–104, 1998.
57. Kenakin T, Agonist-specific receptor conformations. *Trends Pharmacol Sci* **18:** 416–417, 1997.
58. Costa T and Herz A, Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci USA* **86:** 7321–7325, 1989.
59. Costa T, Ogino Y, Munson PJ, Onaran HO and Rodbard D, Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol Pharmacol* **41:** 549–560, 1992.
60. Newman-Tancredi A, Verrièle L, Chaput C and Millan MJ, Labelling of recombinant human and native rat serotonin 5-HT_{1A} receptors by a novel, selective radioligand, [^{3}H]-S15535: Definition of its binding profile using agonists, antagonists and inverse agonists. *Naunyn Schmiedebergs Arch Pharmacol* **357:** 205–217, 1998.
61. Bürgisser E, De Lean A and Lefkowitz RJ, Reciprocal modulation of agonist and antagonist binding to muscarinic cholinergic receptor by guanine nucleotide. *Proc Natl Acad Sci USA* **79:** 1732–1736, 1982.
62. Gether U, Lin S and Kobilka BK, Fluorescent labeling of purified β_2 adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* **270:** 28268–28275, 1995.

63. Gether U, Ballesteros JA, Seifer R, Sanders-Bush E, Weinstein H and Kobilka BK, Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J Biol Chem* **272:** 2587–2590, 1997.
64. Lohse MJ, Molecular mechanisms of membrane receptor desensitization. *Biochim Biophys Acta* **1179:** 171–188, 1993.
65. Su Y-F, Harden TK and Perkins JP, Catecholamine-specific desensitization of adenylate cyclase. Evidence for a multistep process. *J Biol Chem* **255:** 7410–7419, 1980.
66. Hu J, Wang S-Z and El-Fakahany EE, Effects of agonist efficacy on desensitization of phosphoinositide hydrolysis mediated by m1 and m3 muscarinic receptors expressed in Chinese hamster ovary cells. *J Pharmacol Exp Ther* **257:** 938–945, 1991.
67. Whaley B, Yuan N, Birnbaumer L, Clark RB and Barber R, Differential expression of the β-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: A quantitative evaluation. *Mol Pharmacol* **45:** 481–489, 1994.
68. January B, Seibold A, Whaley B, Hipkin RW, Lin D, Schonbrun A, Barber R and Clark RB, β_2-Adrenergic receptor desensitization, internalization, and phosphorylation in response to full and partial agonists. *J Biol Chem* **272:** 23871–23879, 1997.
69. Benovic JL, Staniszewski C, Mayor F Jr, Caron MG and Lefkowitz RJ, β-Adrenergic receptor kinase. Activity of partial agonists for stimulation of adenylate cyclase correlates with ability to promote receptor phosphorylation. *J Biol Chem* **263:** 3893–3897, 1988.
70. Morrison KJ, Moore RH, Carsrud NDV, Trial J, Millman EE, Tuvim M, Clark RB, Barber R, Dickey BF and Knoll BJ, Repetitive endocytosis and recycling of the β_2-adrenergic receptor during agonist-induced steady-state redistribution. *Mol Pharmacol* **50:** 692–699, 1996.
71. Szekeres PG, Koenig JA and Edwardson JM, The relationship between agonist intrinsic activity and the rate of endocytosis of muscarinic receptors in a human neuroblastoma cell line. *Mol Pharmacol* **53:** 759–765, 1998.
72. Stoll L and Muller WE, *In vitro* down-regulation predicts agonist efficacy at central muscarinic cholinergic receptors. *Biochem Pharmacol* **41:** 1963–1966, 1991.
73. Pei G, Samama P, Lohse M, Wang M, Codina J and Lefkowitz RJ, A constitutively active mutant β_2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc Natl Acad Sci USA* **91:** 2699–2702, 1994.
74. Samama P, Pei G, Costa T, Cotecchia S and Lefkowitz RJ, Negative antagonists promote an inactive conformation of the β_2-adrenergic receptor. *Mol Pharmacol* **45:** 390–394, 1994.
75. Westphal RS, Backstrom JR and Sanders-Bush E, Increased basal phosphorylation of the constitutively active serotonin 2C receptor accompanies agonist-mediated desensitization. *Mol Pharmacol* **48:** 200–205, 1995.
76. Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M, Inverse agonist activity of β-adrenergic antagonists. *Mol Pharmacol* **45:** 490–499, 1994.
77. Onaran HO and Costa T, Agonist efficacy and allosteric models of receptor activation. *Ann NY Acad Sci* **812:** 98–115, 1997.