MINIREVIEW

Functional Selectivity through Protean and Biased Agonism: Who Steers the Ship?

Terry Kenakin

Department of Biological Reagents and Assay Development, GlaxoSmithKline Research and Development, Research Triangle Park, North Carolina

Received July 29, 2007; accepted September 27, 2007

ABSTRACT

This article describes functional selectivity of agonists and antagonists and distinguishes conventional cell-based functional selectivity, where the strength of signal produces selective signaling in various organs, from true receptor active-state based selectivity, also alternatively referred to in the literature as "stimulus trafficking," "biased agonism," and "collateral efficacy." This latter mechanism of selectivity depends on the ligand-related conformation of the receptor and is not compatible with the parsimonious view that agonists produce a single receptor active state. In addition, protean agonism is described, whereby a ligand produces positive agonism in quiescent systems and inverse agonism in constitutively active systems. This is a special case of active state-based selectivity in which the ligand produces an active state that is of lower efficacy than the natural constitutively active state. It is postulated that receptor active-state based selectivity, unlike cell-based functional selectivity, is controllable through the chemical structure of the ligand and is therefore more likely to be a viable avenue for therapeutic selectivity in the clinic. Reasons are given for differentiating receptor active-state based selectivity from conventional functional organ selectivity.

There is increasing evidence to show that agonists need not simply be mimics of endogenous neurotransmitters and hormones but rather can cause receptors to exercise only portions of their often vast repertoire of behaviors. In other instances, they can emphasize the interaction of the receptor with certain signaling pathways (Fig. 1A). Likewise, data show that antagonists need not function only as eliminators of function but rather can modulate and otherwise edit endogenous signals (Fig. 1B). These effects have been given various names in different contexts from "stimulus-trafficking," "biased agonism," "collateral efficacy" to a generally accepted "functional selectivity." This latter term, although correct, unfortunately encompasses a breadth of effects described in receptor pharmacology, some of which differ from the concept of receptor-based selectivity. Stimulus-trafficking (Kenakin, 1995a) was originally defined to account for receptor behavior that was incompatible with classic receptor theory, which states that a single receptor active state controls all activation behaviors of a receptor. Pharmacological procedures that use agonist potency ratios to classify agonists and receptors are based on this assumption. However, over the past 10 years, observations that some agonists demonstrate different relative potencies for various cellular pathways (actual reversal of relative potencies can be observed) cannot be reconciled with a single receptor active state and require the involvement of multiple agonist-induced receptor active states.

Single Receptor State Receptor Selectivity

Under certain circumstances, a single receptor active state can lead to functional selectivity (a mechanism differentiated

ABBREVIATIONS: HEK, human embryonic kidney; PACAP, pituitary adenylyl cyclase-activating protein; GRK, G protein-coupled receptor kinase; CCR5, chemokine receptor; ICI118,551, ($\pm$)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; RANTES, regulated on activation normal T cell expressed and secreted; PDZ, postsynaptic density 95/disc-large/zona occludens; ICI174,864, 2[N,N'-diallyl-Tyr1,Aib2,3]Leu5-enkephalin; LP1805, N,N-(2-methylnaphthyl-benzyl)-2-aminoacetonitrile; AMD3100, 1,1'-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride; UK-14304, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine.

in the original definition of stimulus-trafficking as a "strength of signal" mechanism; Kenakin, 1995a). A single activated state can produce selective effects only if the efficiency of coupling is appropriate. Therefore, it is a completely cell-based phenomenon. This well known consequence in single-stimulus systems results in a standard profile whereby a weak partial agonist can produce effect in some (well coupled) tissues and can function as an antagonist (with no direct agonism) in less well coupled tissues. The interplay of weak efficacy with varying levels of affinity can give the overall pattern of organ selectivity, as is seen with the muscarinic agonists carbachol and oxotremorine (see Fig. 2A). Whereas carbachol has low affinity and high efficacy, oxotremorine possesses high affinity and low efficacy. Because potency in well coupled tissues is a complex function of affinity and efficacy and the pEC_{50} of agonists reflects both, no distinction can be made with respect to the relative contribution of affinity or efficacy to overall potency. In contrast, in less well coupled tissues, the maximal response is solely a function of efficacy, whereas the location parameter of the concentration-response curve (pEC_{50}) is solely the function of affinity. Therefore, a dissociation of potency occurs when an agonist shows partial agonism in a tissue versus when it functions as a full agonist in another tissue. As seen in Fig. 2A, oxotremorine and carbachol are both full agonists for contraction of guinea pig ileum; oxotremorine is the more potent agonist. Diminution of the functional muscarinic receptor density with a controlled treatment with the alkylating agent phenoxybenazamine yields a tissue with fewer receptors that is less responsive to muscarinic agonism. As further seen in Fig. 2A, the tissue now only responds to the higher efficacy agonist (carbachol) and shows no effect to oxotremorine. If the two conditions were observed with two tissues of differing sensitivity to muscarinic agonism, a functional selectivity would be concluded.

This mechanism can lead to more complex signaling events for receptors that pleiotropically couple to multiple mechanisms (i.e., G-proteins) in the cell. Under these conditions, the receptor level can control not only the quantity of observed response but also the quality of response. Figure 2B shows the G_i-protein activating effects of increasing cell surface expression of α_2-adrenoceptors on cyclic AMP response; the biphasic response occurs only after sufficient receptor is present to activate both G_i- and G_s-protein (Eason et al., 1992). Similar recruitment of G-protein with increasing receptor density has been shown with calcitonin receptors. In particular, low levels of expression result in solitary coupling to G_s protein [calcitonin activation leads to increased cyclic AMP in human embryonic kidney (HEK) cells]. However, higher levels of receptor expression lead to elevation of cyclic AMP and calcium response mediated by G_q receptors (Kenakin, 1996). Receptor density-linked activation of cellular pathways also has been shown for opioid receptors. In NG108 cells, the opioid agonist [D-Ala^2-D-Leu^5]-enkephalin produces inhibition of adenylate cyclase and stimulation of high-affinity GTPase. Upon reduction of opioid receptor density through alkylation, the less well coupled GTPase response is eliminated and the sole response becomes adenylate cyclase inhibition (Costa et al., 1988). In each of these cases, the agonists involved demonstrate true organ or assay-dependent selectivity. However, this behavior still is consistent with the production of a single receptor active state by the agonists. The strength of the receptor stimulus and the responsiveness of the cell (as controlled either by receptor density and/or efficiency of receptor coupling) combines to produce the demonstrated selective responses. In no instance does the actual rank order of activity reverse; for this to occur, more than one receptor active state must be involved.

Receptor-Based Biased Functional Selectivity

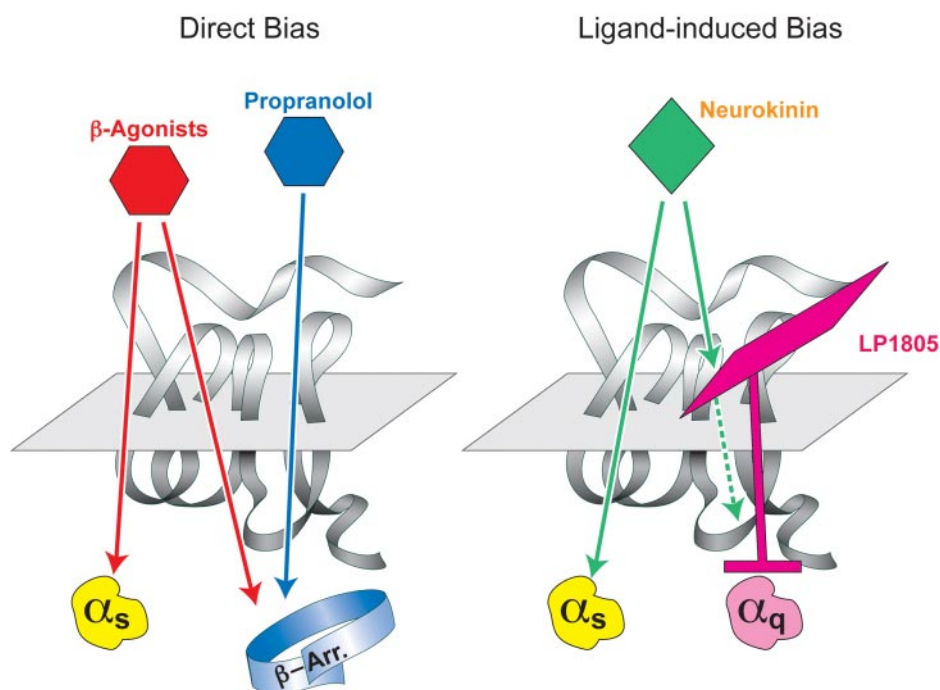Operational theory, as presented by Black and Leff (1983), gives agonist response as:

Fig. 1. Schematic diagram of directly induced and indirectly induced stimulus bias by a ligand. Direct bias is demonstrated by the effects of β-adrenoceptor ligands. Although standard β-adrenoceptor agonists initiate G_s-protein and β-arrestin signaling, the antagonist propanolol does not activate G_s-proteins (in fact it is an inverse agonist for this signaling pathway) but does activate the G-protein independent β-arrestin signaling pathway (data from Azzi et al., 2003; Baker et al., 2003). Indirect imposition of biased signaling occurs when an allosteric ligand cobinds with the agonist to the receptor to modify the signaling properties of the agonist. This is demonstrated by the modulator LP1805 (N,N-(2-methyl naphthyl-benzyl)-2-aminoacetonitrile) which changes the signaling pattern for the endogenous agonist neurokinin A from activation of G_s and G_q protein to only activation of G_s protein. Data from Maillet et al., 2007.

$$\text{Response} = \frac{[A]\tau E_{\max}}{[A](1 + \tau) + K_A} \qquad (1)$$

where K_A is the equilibrium dissociation constant of the agonist-receptor complex (1/affinity), $E_{\max}$ is the maximal response attainable in the system, and τ is a measure of both the efficacy of the agonist and the sensitivity of the system to agonism. The term τ is the receptor density divided by K_E, the equilibrium dissociation constant of the agonist-occupied receptor and the saturable stimulus-response mechanism(s) of the cell. This constant contains both the measure of the overall sensitivity of the cell to agonism and the intrinsic efficacy of the agonist. Thus, a ratio of K_E values (actually τ values with a cancellation of the receptor density term) is a system-independent measure of the relative intrinsic efficacies of the two agonists. It is important to note that for a single receptor active state, the K_E for a given agonist must be constant for all pathways in a cell. The existence of different τ values for various pathways is not compatible with a single receptor active state for that receptor.

A classic hallmark of trafficking of stimulus is the observation of a reversal of relative potencies of full agonists. Eq. 1 can be used to predict the relative potency of full agonists (as the ratio of molar concentration producing 50% maximal response, EC_{50}). For agonists [A1] and [A2], the ratio of EC_{50} values is:

$$\text{Relative Potency} = \frac{K_{A1}(1 + \tau_2)}{K_{A2}(1 + \tau_1)} \qquad (2)$$

From this equation it can be seen that the relative potency depends solely on parameters unique to the agonists and the receptor (namely K_A and τ); thus, it is a system-independent parameter. Therefore, if the agonists produce a single receptor active state, the potency ratio for the production of that state must be constant for all pathways mediated by that active state. The corollary to this is that reversal in the potency ratio for different agonist pathways is incompatible with a single receptor active state. This was the experimental basis for proposing stimulus trafficking on theoretical grounds (Kenakin, 1995a); specifically, Spengler et al. (1993) showed that the order of potency for PACAP agonists PACAP(1–27) and PACAP(1–38) reversed for PACAP-mediated elevated cyclic AMP and inositol phosphate production in LLC-PK1 cells. It was shown that PACAP(1–27) was more potent for the cyclic AMP pathway but that the reverse was true for the inositol phosphate pathway.

Figure 3A gives an example of data that is incompatible with a single receptor active state. In particular, calcium transient responses to two agonists for the human calcitonin receptor are measured in two types of HEK cells: normal, wild-type HEK cells and those cotransfected with $G\alpha_s$ protein. It can be seen that whereas eel calcitonin is more potent than porcine calcitonin in wild-type cells, these agonists reverse their relative potency in cells enriched in $G\alpha_s$ protein. These data indicate that the agonists produce at least two active states, one of which has a higher affinity for the $G\alpha_s$ subunit (Watson et al., 2000). In general, such reversals of full agonist potency are indicators of heterogeneous receptor active states that result in stimulus trafficking.

Another experimental finding that is incompatible with agonist production of identical receptor active states is a difference in the maximal capability of agonists to stimulate
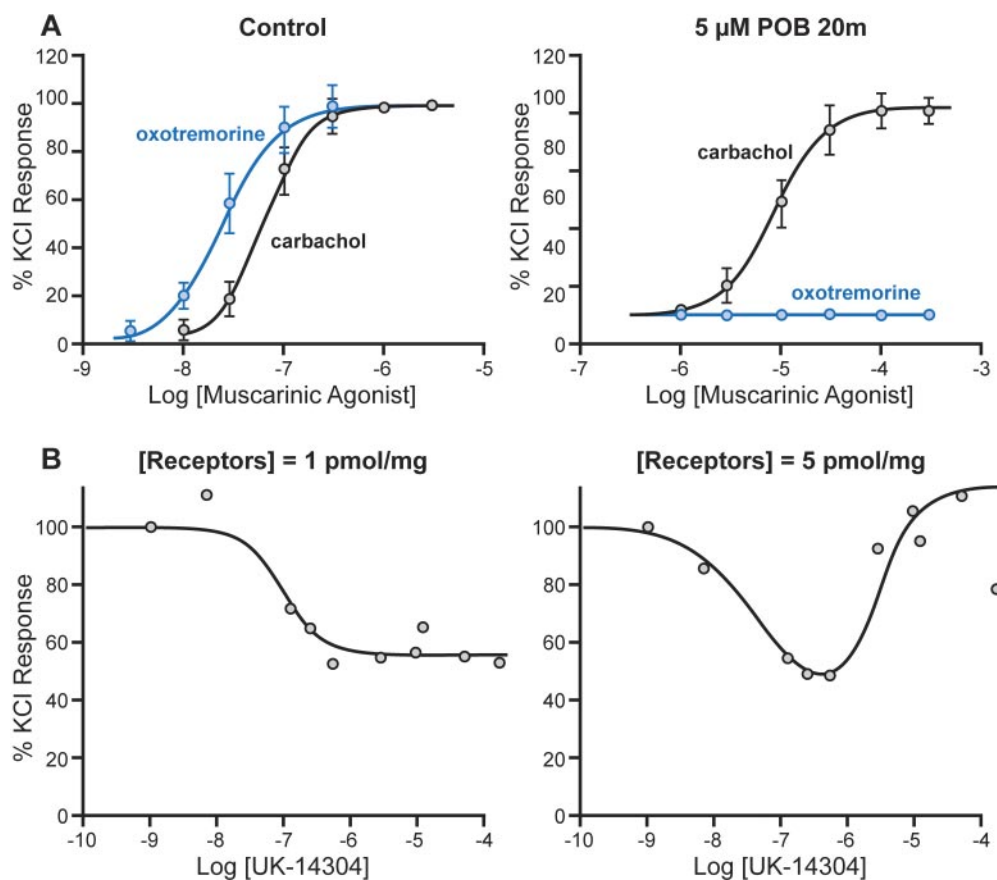

Fig. 2. A, relative potency of muscarinic agonists carbachol and oxotremorine producing contraction of guinea pig isolated ileum. Left, relative effects in untreated ileum; right, effects after exposure to the muscarinic receptor alkylating agent phenoxybenzamine (5 μM for 20 min followed by 1 h of washing). The activation profile changes from oxotremorine > carbachol to carbachol $\gg$ oxotremorine after reduction of muscarinic receptor density. Data from Kenakin (1997). B, effects of the α_2-adrenoceptor agonist UK-14304 on adenylate cyclase in transfected Chinese hamster ovary cells expressing different levels of α_2-C10 receptors. At low expression levels (1 pmol/mg), only G_i-protein-mediated inhibition of adenylate cyclase is observed; at higher receptor levels (5 pmol/mg), a biphasic response is seen with increases due to activation of G_s-protein. New graphs from the data of Eason et al. (1992).

different pathways in cells. From eq. 1, the relative maximal responses for two agonists [A1] and [A2] (i.e., as [A]→∞) is calculated as:

$$\text{Relative maxima} = \frac{\tau_1(1 + \tau_2)}{\tau_2(1 + \tau_1)} \quad (3)$$

It can be seen from this equation that the relative maximum is strictly a function of the efficacy of the agonist. If, for two given agonists, the ratio of the relative maxima is >1 ($\text{Max}_{A1} > \text{Max}_{A2}$), then it can be shown that $\tau_1 > \tau_2$. A change in the relative maximum would necessitate a change in the relative efficacy of the agonists (i.e., a change in the nature of the agonist-activated receptor producing response). Therefore, a reversal of relative maximal responses for two pathways for any two agonists is incompatible with a single receptor active state and strongly indicates that the two agonists produce different primary active states (i.e., true receptor-based functional selectivity). Figure 3B shows an example of such a reversal of the maximal capabilities of serotonin agonists for arachidonic acid and inositol triphosphate production in response to activation of the 5-HT$_{2C}$ receptor (Berg et al., 1998). This effect is incompatible with the idea that theses two agonists produce the same receptor active state for the activation of these cellular pathways.

Protean Agonism

A special case of receptor-based functional selectivity is "protean" agonism. These are ligands that produce a receptor active state that is capable of initiating signal where there is none but from a receptor active state that is less efficacious than the naturally occurring, spontaneously formed constitutive active state (Kenakin,1995b, 2001). In a quiescent system, consisting mainly of receptors in the inactive state, protean agonists produce positive agonism. In contrast, in a constitutively active system consisting of a substantial amount of spontaneously formed receptor active state, protean agonists produce inverse agonism. This is because they convert the efficacious active state to a less efficacious ligand-selective active state (see Fig. 4). Because the ligand effect changes in response to the system, these molecules were named after the Greek sea-god Proteus (son of Poseidon), who could change shape at will depending on his environment and needs (Kenakin, 1995b). Examples of protean agonists, such as dichloroisoproterenol, have been seen experimentally (Chidiac et al., 1996). Protean agonists theoretically should be the best equalizers of endogenous effect because they would reduce effects due to endogenous agonist tone and effects due to constitutive activity; the latter activity would
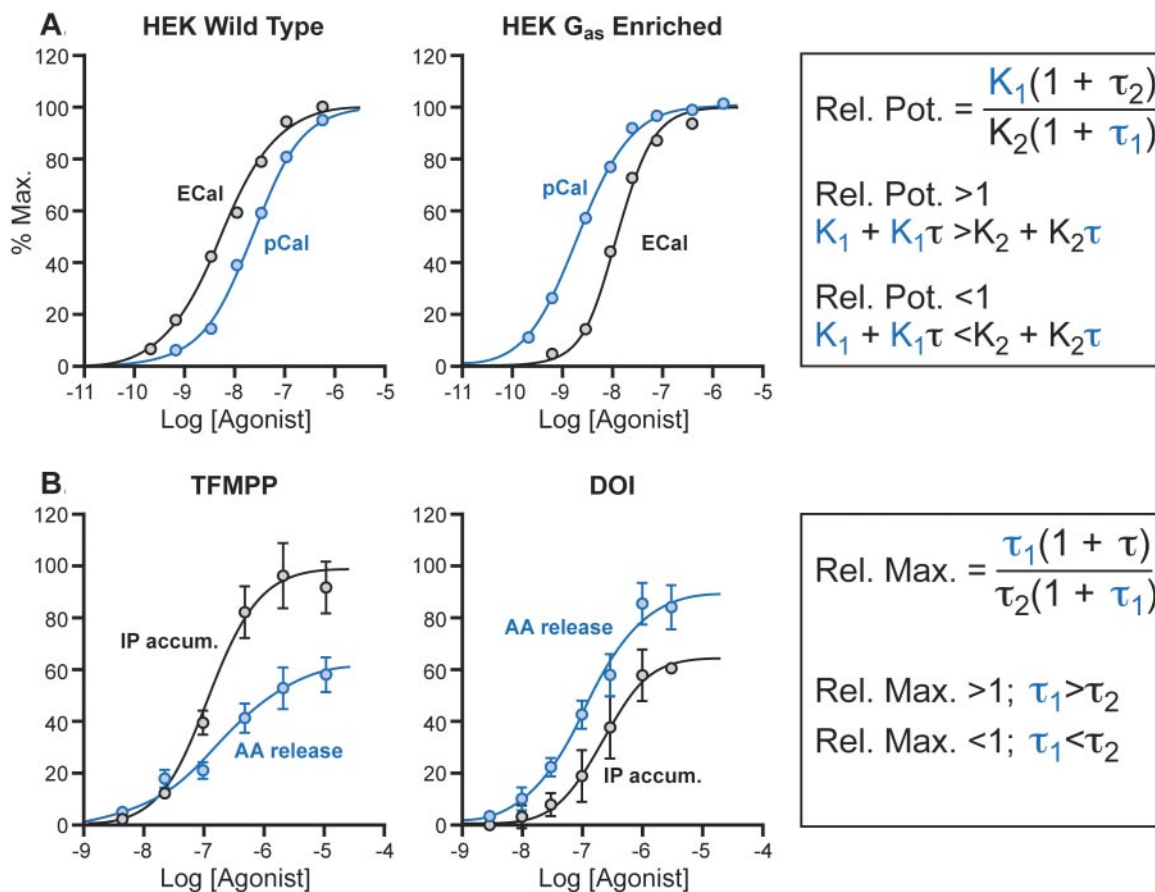

Fig. 3. Reversal of potencies of agonists not compatible with production of a single uniform receptor active state. A, relative potency of eel and porcine calcitonin (calcium response in HEK cells transfected with human calcitonin receptors) in wild-type cells and cells cotransfected with Gα$_s$-protein. It can be seen that enrichment of the Gα$_s$-protein selectively increases the potency of porcine calcitonin to the point where the relative potencies of these full agonists is reversed. New graphs from the data of Watson et al. (2000). B, reversed maximal capability of agonists to produce arachidonate release and inositol phosphate (IP) accumulation through activation of 5-HT$_{2C}$ receptors in Chinese hamster ovary cells. Although 3-trifluoromethylphenyl-piperazine (TFMPP) has greater efficacy for IP accumulation than arachidonate release, the reverse is true for (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). This type of reversal clearly indicates that these agonists produce different receptor active states. New graphs from the data of Berg et al. (1998).

not be observed with normal partial agonists because a partial agonist would be incapable of altering the constitutive receptor activity. It is important to note that the term protean refers to a specialized receptor ligand and not just a generic functionally selective ligand.

Indirect Ligand-Induced Bias

Orthosteric antagonists occlude the agonist binding site, thereby preventing receptor activation. No "texture" in the antagonism is possible with this mechanism because the result is an unresponsive receptor. This is not necessarily true of an allosteric modulator that binds to its own site on the receptor and allows the agonist to bind as well. With this mechanism, the modulator may modify the response to agonist with a range of effects from complete inhibition to potentiation. The effect is caused by a modulator-induced change in the conformation of the receptor; i.e., the modulator stabilizes an allosteric conformation that has modified responsiveness to the agonist. The change in conformation of the receptor also may modify the interaction of the receptor with cellular membrane interactants such as G-proteins, GRKs, β-arrestin(s) and other proteins. Because these molecules bind to different loci on the receptor, the changes in responsiveness need not be uniform (in fact, it might be predicted that the changes should not be). This idea was the basis for using different G-protein enrichment to detect agonist-selective receptor active states shown in Fig. 3A. Because different regions of the receptor were known to interact with various G-proteins, the postulate was that diverse receptor conformations of the receptor would not expose these regions in an identical manner and that the heterogeneity of exposure with dissimilar states would be reflected in variable reliance of response on different G-proteins (this is a case of using the G-protein complement of the cell to detect different conformations). As seen in Fig. 3A, this was confirmed as ligand-directed stimulus trafficking was made obvious by diversity in G-protein content of cells. The corollary to this idea, then, is that a change in conformation will not present
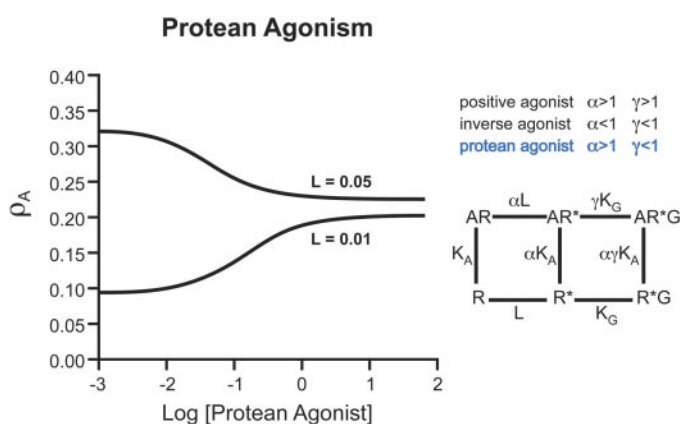
identical changes to different signaling partners for the receptor in the cell. Under these circumstances, an allosteric change in receptor conformation could alter the array of responses produced by the agonist (Fig. 1B). For example, neurokinin produces activation of G_s and G_q protein through NK1 receptors. However, the allosteric modulator LP1805 changes this pattern to one of *enhanced* G_q response and *antagonism* of G_s activation (Maillet et al., 2007). Likewise, prostaglandin D2 interacts with CRTH2 receptors to activate G_i-protein and β-arrestin. Binding of the modulator sodium tosyltryptophan causes PDG2 to lose its ability to initiate receptor interaction with β-arrestin but not G_i-protein (Mathiesen et al., 2005). In both of these cases, the allosteric modulator imposes collateral efficacy (partial expression of all possible receptor behaviors) onto the natural agonist.

Receptor-Based Selectivity

There have been reviews citing many instances of true receptor-based selectivity of trafficking with respect to signaling pathways in cells (Kenakin, 2002a, 2003, 2006; Perez and Karnik, 2005; Urban et al., 2007). The basic difference between this and cell-based selectivity is that the ligand "steers the ship" from the point of view of controlling the effect. In contrast, cell-based selectivity relies on the stoichiometry and sensitivity of the cellular components driving the response. Although any given system can yield therapeutically favorable instances of selectivity, the effect is still under the control of cell physiology and pathology. Because drugs usually are developed in cell systems not controlled by pathological mechanisms and then used in systems that are, it is difficult to correlate cell-based selectivity seen in test systems with corresponding selectivity in the therapeutic system (i.e., there may or may not be correspondence).

A better starting point for the design of therapeutically useful functionally selective drugs is to have the ligand itself direct the stimulus. Under these circumstances, selectivity occurs in all systems with no dependence on the relative emphasis that any given cell places on a signaling pathway. The control of the effect is governed by the differential affinities of the ligand-bound receptor for various cellular pathway effectors. Thus, the activated receptor will ignore some pathways and preferentially activate others. From this standpoint, receptor-based selectivity is unique and should be differentiated from general functional selectivity.

It is useful to mathematically model receptor coupling to extend predictions to multiple effector systems. Thus, a receptor that binds a ligand [A] to form a ligand-bound complex [AR] can go on to bind to any number of "effectors" in the cell (designated E_1 to E_n); these range from different G-proteins to β-arrestin(s) and GRKs. The receptor is conserved in that the limiting constraint on the system is the amount of receptor available to couple to the cellular components; this allows for the modeling of the effects of changing receptor density. Finally, the response can be given as a logistically forced function of the effector complex (for example, for effector E_i, the complex ARE_i goes on to stimulate a pathway in the cell that leads to response of the form $([ARE_i]/([ARE_i] + \varphi_i)$, where φ_i is a fitting parameter). The addition of this function does not alter the conclusions made from this model but does eliminate the necessity of assuming a one-to-one relationship between response and amount of effector complex. It can be

Protean Agonism

Fig. 4. Molecular description of Protean agonism. Ligands that enrich existence of the active state R* (through α and $\gamma > 1$) produce positive agonism. Likewise, ligands that destabilize R* and shift equilibria toward R (α and $\gamma < 1$) will produce inverse agonism. Ligands that enrich an active state ($\alpha > 1$) that has a reduced affinity for the G-protein than the spontaneously formed active state ($\gamma < 1$) will produce agonism in systems not containing spontaneous R* (will be agonists in nonconstitutively active systems). However, this ligand will produce a receptor species less likely than R* to induce response and therefore will be an inverse agonist in constitutively active systems.

shown that the fractional response for a pathway (designated pathway i) is given by (derived in *Appendix*):

$$\text{Resp}_i = \frac{[A]/K_A\ \tau_{Ai}\ [E_i]}{[A]/K_A(\tau_{Ai}[E_i] + \varphi_i(1 + \sum_{i=1}^{n} [E_i]/K_{Ei})) + \varphi_i} \quad (4)$$

where the size of the effector pool is given by $[E_i]$, and τ_{Ai} is the efficacy of the agonist for the response pathway. This model allows the prediction of the effects of ligands that produce a single versus multiple active states in systems of varying receptor density and/or receptor coupling efficiencies when there are a number of effectors coupling to the receptor.

In comparing two agonists, the ratio of degree of stimulation of a given pathway (for equivalent values of $[A]/K_A$), can be calculated with equations analogous to eqs. 2 and 3 for relative potency and relative maxima (see *Appendix*). Thus, the relative potency of agonists A and B in a multieffector system is given by:

$$\text{Potency Ratio} = \frac{K_B\ (\tau_{Ai} + \vartheta)}{K_A\ (\tau_{Bi} + \vartheta)} \quad (5)$$

given:

$$\vartheta = \frac{\varphi_i(1 + \sum_{i=1}^{n} [E_i]/K_{Ei})}{[E_i]} \quad (6)$$

where ϑ is a tissue constant for all agonists in any given tissue. This term relates to the particular cellular milieu of coupling mechanisms available to the receptor. The ratio of maxima of the agonists for any pathway is given by:

$$\text{Relative Max.} = \frac{\tau_{Ai}\ (\tau_{Bi} + \vartheta)}{\tau_{Bi}\ (\tau_{Ai} + \vartheta)} \quad (7)$$

It can be seen from an examination of eqs. 5 and 7, for any given pathway i, if the ratio is measured to be >1, this can occur only if $\tau_{Ai} > \tau_{Bi}$. Therefore, a reversal of the relative potency or maximal responses can occur only if the relative efficacies for that pathway reverse (i.e., $\tau_{Ai} < \tau_{Bi}$). Because $\tau = [R_{tot}]/K_{Ei}$ and $[R_{tot}]$ is constant for both agonists, such a reversal can occur only if the equilibrium dissociation constant of the agonist-occupied receptor changes. If this is observed, it would indicate a change in the nature of the agonist-activated receptor (i.e., a different receptor active state). It should also be noted that cell-based selectivity would depend only on values of ϑ for cells and is not controllable through the structure of the agonist.

Should Functional Selectivity Be an Expected Event?

Seven-transmembrane receptors are allosteric proteins and are therefore capable of adopting different conformations. An important outcome of this behavior is that the changes in conformation can be global in nature; i.e., changes in numerous regions of the protein may occur simultaneously. Thus, a modulator may stabilize one or more preexisting but possibly rare conformations of the receptor, and those may show altered positions of amino acids in numerous

locations. It is useful to think about this type of effect in terms of receptor active states; a starting point for this is to consider the nature of a seven-transmembrane receptor active state.

A receptor active state interacts with a membrane component to elicit a change in cellular behavior. Thus, a change in the receptor conformation presumably opens the door to the binding of the receptor to an effector protein such as a G-protein or β-arrestin. For example, an 11-amino acid peptide sequence from the C-terminal region of the third intracellular loop of the β-adrenoceptor (Thr284–Thr291) has been shown to have the unique ability to initiate G_s-mediated adenylate cyclase activation in turkey erythrocytes (Münch et al., 1991). This suggests that conformations that expose this region of the receptor will cause cytoplasmic signaling. The corollary to this idea is that conformations of the receptor that prevent exposure of this region to G_s-protein will be inactive and not signal. This predicts the existence of numerous "active" and "inactive" conformations [referred to as an "ensemble" (Kenakin, 2002b)]. The existence of multiple states is supported by point mutation studies carried out on the α_{1B}-adrenoceptor in which it was found that amino acid substitution at position 293 of the receptor produces a constitutively active receptor state. It is noteworthy that substitution of 20 different amino acids in this location resulted in 20 different levels of constitutive activity, indicating 20 different conformations capable of signaling (Kjelsberg et al.,1992) and a low level of fidelity with respect to the conformational requirements for activation.

Seven-transmembrane receptors can also demonstrate the allosteric trait of probe dependence. For example, the CXCR4 receptor antagonist AMD3100 and antibody P140 block chemotaxis produced by the natural CXCR4 agonist stromal-derived factor 1-α. However, these antagonists have no effects at all on the response to the stromal-derived factor 1-α peptide fragments [Ala-Ser-Leu-Trp] and [Arg-Ser-Val-Met] (Sachpatzidis et al., 2003). Such probe dependence would be predicted to be amplified in systems in which different regions of the receptor mediate the affinities (and efficacies) of the probes. Thus, cells in which different regions of the receptor interact with different G-proteins (i.e., see Ikezu et al., 1992) define sensitive systems to detect differences in receptor conformation. The basis for this expectation is the notion that different tertiary protein conformations would not be expected to produce identical movements of these different intracellular loops and that these differences would be detected by cytosolic interactants with the receptor (see Fig. 5). In fact, this has been shown to be the case. For example, the CB_1 cannabinoid ligand desacetyllevonantradol, a *positive* agonist for G_{i1} and G_{i2}, is an *inverse* agonist for G_{i3}. Likewise, (*R*)-methanandamide is an *inverse* agonist for G_{i1} and G_{i2} and a *positive* agonist for G_{i3} (Mukhopadhyay and Howlett, 2005). A logical interpretation of these data is to postulate that the receptor conformations stabilized by these ligands produce different changes in the various regions interacting with these G-proteins to produce heterogeneous effects (i.e., classical allosteric probe dependence).

Binding profiles for antibodies also can be sensitive indicators of tertiary conformations of specific regions of receptors. For example, it has been shown that allosteric modulators of the chemokine receptor CCR5 produce different binding profiles for various antibodies to the receptor

(Kenakin, 2007). These data are consistent with the notion that a modulator can produce different conformational effects in various regions of receptors. If these regions interact with cellular signaling mechanisms, this could translate into differences in receptor signaling.

Biased agonism and receptor-based functional selectivity was first defined in systems in which 7Transmembrane receptors interacted with multiple G-proteins in a pleiotropic manner (Kenakin, 1995a; Lawler et al., 1999). However, a new paradigm for 7Transmembrane receptor signaling has been defined in the form of G-protein-independent, β-arrestin-mediated signaling (Lefkowitz, 2004, 2006; Terrillon and Bouvier, 2004; Lefkowitz and Shenoy, 2005; Luttrell, 2005); this sets the stage for further multiple receptor region allosteric dependence. Thus, although β-adrenoceptor blocking agents such as atenolol and bisoprolol are inverse agonists for Gs-protein- and β-arrestin-mediated extracellular signal-regulated kinase activation, others, such as ICI118,551 and propranolol, are inverse agonists for Gs-protein and *positive* agonists for the extracellular signal-regulated kinase pathway (Azzi et al., 2003; Baker et al., 2003; Galandrin and Bouvier, 2006). Likewise, agonists for the chemokine CCR5 receptor RANTES and AOP-RANTES both produce CCR5-mediated calcium response (Proudfoot et al., 1999) and both induce receptor phosphorylation. However, aminooxypentane-RANTES functions as a "superagonist" of phosphorylation, producing 300% maximal effect of RANTES (Oppermann et al., 1999), which indicates differential effects on the sites on CCR5 responsible for calcium signaling and GRK binding.

Site-directed mutagenesis studies suggest that the interaction of receptors with β-arrestin are complex, involving a large number of surface charges, and that elements of arrestin are differentially engaged by various functional forms of the receptor (Charest et al., 2005; Hanson and Gurevich, 2006). This idea, when coupled to the fact that receptors have been shown directly to adopt different conformations in response to ligand-binding (Gether et al., 1995; Ghanouni et al., 2001; Palanche et al., 2001, Yao et al., 2006; Swaminath et al., 2004), suggests that not all ligands that cause engagement with β-arrestin will do so in a uniform manner. Furthermore, because the β-arrestin/receptor complex can internalize and function as a signaling scaffold for mitogen-activated protein kinases (receptosomes), it is as yet unclear whether the changes in the conformation of β-arrestin in this process (Xiao et al.,

2004) are sensitive to the type of ligand bound to the receptor.

Heterogeneous probe dependence would be expected to increase with increasing numbers of receptor probes; in this case, "probe" refers to the cellular interactants coupling to the receptor to initiate cellular response. The list of such probes is increasing, ranging from different G-proteins [the thyrotropin receptor has been shown to interact with all four G-protein families (Laugwitz et al., 1996)] to β-arrestin(s), GRKs, receptor activity-modifying proteins, PDZ proteins, and numerous other membrane-bound and cytosolic interactants (Bockaert and Pin, 1999; Brady and Limbird, 2002; Bockaert et al., 2004; Gavarini et al., 2006). Each of these interactions is defined by a distinct affinity equilibrium constant. Therefore, multiple receptor conformations would not be expected to produce uniform multiple relative propensities to activate different signaling pathways; i.e., different conformations would be expected to produce functional selectivity.

It is premature to conclude whether the theoretical prediction of widespread functional selectivity will be the exception or the rule with different agonists. This should become clearer with the accumulation of more data with new synthetic agonists. This is analogous to the situation encountered with the discovery of inverse agonism for the opioid receptor ligand ICI17864. Although this seemed to be an exception when first observed (in apparent disagreement with the theoretical prediction that it should be the rule), the subsequent widespread testing of antagonists in constitutively active systems confirmed that inverse agonism is the rule, not the exception. One estimate showed that approximately 85% of orthosteric antagonists were inverse agonists. This is in agreement with theoretical prediction indicating that identical affinities for different receptor conformations would not be expected (Kenakin, 2004). It will be interesting to determine whether the ability to run high-throughput screens to detect synthetic agonists in functional screening mode (as opposed to binding mode) will subsequently increase the number of agonists (both orthosteric and allosteric) available to study selective pathway stimulation. In addition, just as the availability of constitutively active systems enabled studies to address the prevalence of inverse agonism, the availability of assays that independently measure various aspects of receptor function (i.e., G-protein, β-arrestin interaction, internalization, and phosphorylation) will un-
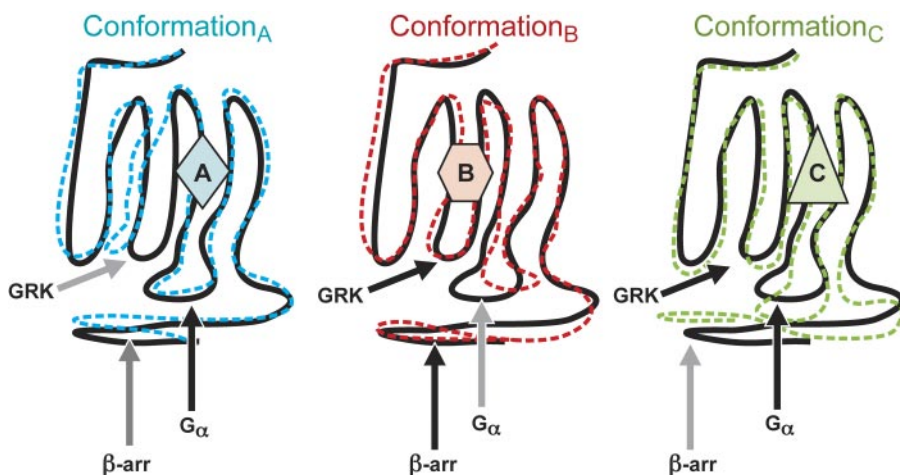
Fig. 5. Schematic diagram depicting levels of conformational aberration produced in different areas of the receptor upon stabilization of receptor conformations by different agonists. Arrows depict various regions of interaction of the receptor with cytosolic interactants such as different G-proteins and β-arrestin. It might be surmised that dissimilar conformations affect these various regions to varying degrees causing respective differences in effect for diverse coupling mechanisms.

cover selectivity in molecules previously thought to uniformly mimic natural agonists.

Conclusions

The identification of receptor-based functional selectivity is a useful endeavor in terms of using the chemical structure of the ligand as a control point to induce selective cellular function. From this standpoint, it is important to identify true receptor-based selectivity and differentiate it from general selectivity that can be obtained courtesy of the wiring in any given cell type. Therefore, true reversal of potency ratio or maximal response should be the hallmark for differentiating these functional selectivities, and accurate nomenclature of the result should follow to avoid confusion in the literature.

Appendix

The model consists a single receptor bound by a ligand A that can interact with various elements in the cell membrane after activation according to the operational model of agonism (Black and Leff, 1983). The amount of ligand-bound receptor is given by mass action.

$$[AR] = [A][R]K_a \tag{8}$$

where K_a is the equilibrium association constant of the ligand-receptor complex. The subsequent complex with an interactant E_i is given by:

$$[ARE_i] = [AR][E_i]K_{ei} = [A][R]K_a[E_i]K_{ei} \tag{9}$$

where K_{ei} is the equilibrium association constant of the ternary ARE_i complex.

The receptor conservation equation for all of the receptor species for all membrane interactants is given by:

$$[R_{tot}] = [AR] + [R] + [ARE_1] + [ARE_2]...+...[ARE_n] \tag{10}$$

It can be shown that the fraction of receptor bound to any one reactant is given by $\rho_{AEi} = [ARE_i]/[R_{tot}]$. For $\rho_{AE1} = [ARE_1]/[R_{tot}]$ for a system with n receptor interactants:

$$\rho_{AE1} = \frac{[A]/K_A[E_1]/K_{E1}}{[A]/K_A (1+\sum_{i=1}^{n}[E_i]/K_{Ei}) + 1} \tag{11}$$

where K_A, K_{E1} and K_{Ei} are equilibrium dissociation constants ($1/K_A$, $1/K_{E1}$, and $1/K_{Ei}$, respectively).

The stimulus-response pathway(s) producing response from the activation of each pathway controlled by the interactants is modeled by a simple logistic input-output forcing function of the form:

$$Response_i = [Input_i]/([Input_i] + \varphi_i) \tag{12}$$

where φ_i is a parameter describing the efficiency of the coupling process (relationship between $[ARE_i]$ and the rest of the stimulus response mechanism of the cell). The input for this function is the number of receptors bound by the ligand and coupled to the particular process given by $\rho_{AEi} [R_{tot}]$. Substituting this into eq. 11 yields:

$$Resp_i = \frac{\rho_{AEi}[R_{tot}]}{\rho_{AEi}[R_{tot}] + \varphi_i} \tag{13}$$

Substituting for ρ_{AEi} yields:

$$Resp_i = \frac{[A]/K_A[E_i]/K_{Ei}[R_{tot}]}{[A]/K_A([E_i]/K_{Ei}[R_{tot}] + \varphi_i(1 + \sum_{i=1}^{n}[E_i]/K_{Ei})) + \varphi_i} \tag{14}$$

For an agonist A, substituting τ_{Ai} for $[R_{tot}]/K_{Ei}$ yields:

$$Resp_i = \frac{[A]/K_A \, \tau_{Ai}[E_i]}{[A]/K_A(\tau_{Ai}[E_i] + \varphi_i(1 + \sum_{i=1}^{n}[E_i]/K_{Ei})) + \varphi_i} \tag{15}$$

It is useful to define the following cell specific term:

$$\vartheta = \frac{\varphi_i(1 + \sum_{i=1}^{n}[E_i]/K_{Ei})}{[E_i]} \tag{16}$$

which causes eq. 15 to be rewritten as:

$$Resp_i = \frac{[A]/K_A \, \tau_{Ai}[E_i]}{[A]/K_A \, (\tau_{Ai} + \vartheta) + \varphi_i} \tag{17}$$

It can be seen from this equation that the observed potency of agonist A for the response pathway is given by:

$$K_{obs} = \frac{K_A\varphi_i}{(\tau_{Ai} + \vartheta)} \tag{18}$$

For two agonists A and B, the potency ratio is

$$Potency\ Ratio = \frac{K_B \, (\tau_{Ai} + \vartheta)}{K_A \, (\tau_{Bi} + \vartheta)} \tag{19}$$

Likewise, the maximal response from eq. 17 is:

$$Max.\ Resp. = \frac{E_i\tau_{Ai}}{(\tau_{Ai} + \vartheta)} \tag{20}$$

The relative maximal responses to agonists A and B are then:

$$Relative\ Max. = \frac{\tau_{Ai} \, (\tau_{Bi} + \vartheta)}{\tau_{Bi} \, (\tau_{Ai} + \vartheta)} \tag{21}$$

References

Azzi M, Charest PG, Angers S, Rousseau G, Kohout (2003) β-Arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G-protein-coupled receptors. *Proc Natl Acad Sci U S A* **100:**11406–11411.

Baker JG, Hall IP, and Hill SJ (2003) Agonist and inverse agonist actions of β-blockers at the human β₂-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol* **64:**1357–1369.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, and Clarke WP (1998) 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.

Black JW and Leff P (1983) Operational models of pharmacological agonist. *Proc R Soc Lond Biol* **220:**141–162.

Bockaert J and Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary process. *EMBO J* **18:**1723–1729.

Bockaert J, Dumuis A, Fagni L, and Marin P (2004) GPCR-GIP networks: a first step in the discovery of new therapeutic drugs? *Curr Opin Drug Discov Devel* **7:**649–657.

Brady AE and Limbird LE (2002) G-protein coupld receptor interacting proteins: emerging roles in localization and signal transduction. *Cell Signal* **14:**297–309.

Charest PG, Terrillon S, and Bouvier M (2005) Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO Reports* **6**:334–340.

Chidiac P, Nouet P, vBouvier M (1996) Agonist-induced modulation of inverse agonist efficacy at the β₂-adrenergic receptor. *Mol Pharmacol* **50**:662–666.

Costa T, Klinz FJ, Vachon L, and Herz A (1988) Opioid receptors are coupled tightly to G proteins but loosely to adenylate cyclase in NG108-15 cell membranes. *Mol Pharmacol* **34**:744–754.

Eason MC, Kurose H, Holt BD, Raymond JR, and Liggett SB (1992) Simultaneous coupling of α₂-adrenergic receptors to two G-proteins with opposing effects. *J Biol Chem* **267**:15795–15801.

Galandrin S and Bouvier M (2006) Distinct signaling profiles of β₁ and β₂ adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**:1575–1584.

Gavarini S, Becamel C, Altier C, Lory P, Poncet J, Wijnholds J, Bockaert J, and Marin P (2006) Opposite effects of PSD-95 and MPP3 PDZ proteins on serotonin 5-hydroxytryptamine(2C) receptor desensitization and membrane stability. *Mol Biol Cell* **17**:4619–4631.

Gether U, Lin S, and Kobilka BK (1995) Fluorescent labeling of purified β₂-adrenergic receptor: evidence for ligand specific conformational changes. *J Biol Chem* **270**:28268–28275.

Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR et al (2001) Functionally different agonists produce distinct conformations in G-protein coupling domains of the β₂-adrenergic receptor. *J Biol Chem* **276**:24433–24436.

Hanson SM and Gurevich VV (2006) The differential engagement of arrestin surface charges by the various functional forms of the receptor. *J Biol Chem* **281**:3458–3462. 16339758

Ikezu T, Okamoto T, Ogata E, and Nishimoto I (1992) Amino acids 356–372 constitute a Gᵢ-activator sequence of the α2-adrenergic receptor and have a Phe substitute in the G-protein-activator sequence motif. *FEBS Lett* **311**:29–32.

Kenakin TP (1995a) Agonist-receptor efficacy II: agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**:232–238.

Kenakin TP (1995b) Pharmacological Proteus? *Trends Pharmacol Sci* **16**:256–258.

Kenakin TP (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* **48**:413–463.

Kenakin TP (1997) *The Pharmacologic Analysis of Drug Receptor Interaction*, 3rd ed, Lippincott-Raven, New York.

Kenakin TP (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15**:598–611.

Kenakin TP (2002a) Efficacy at G protein coupled receptors. *Annu Rev Pharmacol Toxicol* **42**:349–379.

Kenakin TP (2002b) Efficacy at G protein coupled receptors. *Nat Rev Drug Discov* **1**:103–110.

Kenakin TP (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24**:346–354.

Kenakin TP (2004) Efficacy as a vector: the prevalence and paucity of inverse agonism. *Mol Pharmacol* **65**:2–11.

Kenakin TP (2006) Collateral efficacy as pharmacological problem applied to new drug discovery. *Exp Opin Drug Discov* **1**:635–652.

Kenakin TP (2007) Collateral efficacy in drug discovery: taking advantage of the good (allosteric) nature of 7TM receptors. *Trends Pharmacol Sci* **28**:407–415.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG, and Lefkowitz RJ (1992) Constitutive activation of the α₁B-adrenergic receptor by all amino acid substitutions at a single site. *J Biol Chem* **267**:1430–1433.

Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, and (1996) The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci U S A* **93**:116–120.

Lawler CP, Prioleau C, Lewis MM, Mak C, Jiang D, Schetz JA, Gonzalez AM, Sibley DR, and Mailman RB (1999) Interactions of the novel antipsychotic aripiprazole (OPC-14597) with dopamine and serotonin receptor subtypes. *Neuropsychopharmacology* **20**:612–627.

Lefkowitz RJ (2004) Historical review: a brief history and personal retrospective of seven-transmembrane receptors. *Trends Pharmacol Sci* **25**:413–422.

Lefkowitz RJ and Shenoy SK (2005) Transduction of receptor signals by β-arrestins. *Science* **308**:512–517.

Lefkowitz RJ, Rajagopal K, and Whalen EJ (2006) New roles for beta-arrestins in cell signaling: not just seven-transmembrane receptors. *Mol Cell* **24**:643–652.

Luttrell LM (2005) Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. *J Mol Neurosci* **26**:253–263.

Maillet EL, Pellegrini N, Valant C, Bucher B, Hibert M, Bourguignon JJ, and Galzi JL (2007) A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. *FASEB J* **21**:2124–2134.

Mathiesen JM, Ulven T, Martini L, Gerlach LO, Heinemann A, and Kostenis E (2005) Identification of indole derivatives exclusively interfering with a G protein-independent signaling pathway of the prostaglandin D2 receptor CRTH2. *Mol Pharmacol* **68**:393–402.

Mukhopadhyay S and Howlett AC (2005) Chemically distinct ligands promote differential CB₁ cannabinoid receptor-Gᵢ protein interactions. *Mol Pharmacol* **67**:2016–2024.

Münch G, Dees C, Hekman M, and Palm D (1991) Multisite contacts involved in coupling of the β-adrenergic receptor with the stimulatory guanine-nucleotide-binding regulatory protein. *Eur J Biochem* **198**:357–364.

Oppermann M, Mack M, Proudfoot AEI, and Olbrich H (1999) Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus. *J Biol Chem* **274**:8875–8885.

Palanche T, Ilien B, Zoffmann S, Reck MP, Nucher B, Edelstein SJ et al (2001) The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. *J Biol Chem* **276**:34853–34861.

Perez DM and Karnik SS (2005) Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev* **57**:147–161.

Proudfoot AEI, Buser R, Borlat F, Alouani S, Soler D, Offord RE, Scroder, J.-M, Power CA, and Wells TNC (1999) Amino-terminally modified RANTES analogues demonstrate differential effects on RANTES receptors. *J Biol Chem* **274**:32478–32485.

Sachpatzidis A, Benton BK, Manfredi JP, Wang H, Hamilton A, Dohlman HG *et al.* (2003) Identification of allosteric peptide agonists. *J Biol Chem* **278**:896–907.

Spengler D, Waber C, Pantolini C, Hosboer F, Bockaert J, Seeburgt PH, and Journot L (1993) Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365**:170–175.

Swaminath G, Xiang Y, Lee TW, Steenhuis J, Parnot C, and Kobilka BK (2004) Sequential binding of agonists to the β₂-adrenoceptor: B2 kinetic evidence for intermediate conformation states. *J Biol Chem* **279**:686–691.

Terrillon S and Bouvier M (2004) Receptor activity-independent recruitment of β-arrestin reveals specific signaling modes. *EMBO J* **23**:3950–3961.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, et al (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1–13.

Watson C, Chen G, Irving PE, Way J, Chen W-J, and Kenakin TP (2000) The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Mol Pharmacol* **58**:1230–1238.

Xiao K, Shenoy SK, Nobles K, and Lefkowitz RJ (2004) Activation-dependent conformational changes in β-arrestin 2. *J Biol Chem* **279**:55744–55753.

Yao X, Parnot C, Deupi X, Ratnala VRP, Swaminath G, Farrens D, and Kobilka B (2006) Coupling ligand structure to specific conformational switches in the β₂-adrenoceptor. *Nat Chem Biol* **2**:417–422.

Address correspondence to: Terry Kenakin, Department of Biological Reagents and Assay Development, GlaxoSmithKline Research and Development, 5 Moore Drive, Research Triangle Park, NC 27709. E-mail: terry.p.kenakin@gsk.com