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The effect of the molecular mechanism of G protein-coupled receptor activation on the process of signal transduction

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Abstract

A thermodynamic model of signal transduction that incorporates the possibility of multiple conformational states between the inactive and the active forms of the receptor was developed. The obtained equilibrium model is equivalent to the extended ternary complex of Samama et al. (J. Biol. Chem. 268 (1993) 4625–4636) if only two states of the receptor exist. These multiple equilibria between receptor states are modeled by two sets of equilibrium constants: $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$, in the presence of the ligand; and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$, in the absence of the ligand. The higher the value of these constants, the more efficiently the active form of the receptor is generated. Intrinsic efficacy of the agonist is defined in the present formulation as the molecular processes induced by ligands in the receptor that lead to the active form of the receptor. Both the energetics (associated to $K_{\Pi AR}$) and mechanism of the process of receptor activation (associated to $K_{\Sigma\Pi AR}$) are important in eliciting the maximum response. Moreover, analytical expressions of basal activity, potency and maximum response were obtained. These definitions were used to classify the extra cellular ligand as agonists ($K_{\Sigma\Pi AR} > K_{\Sigma\Pi R}$), inverse agonists ($K_{\Sigma\Pi R} > K_{\Sigma\Pi AR} > 0$), neutral antagonists ($K_{\Sigma\Pi AR} = K_{\Sigma\Pi R}$), and pure antagonists. © 1997 Elsevier Science B.V.

Keywords: Signal transduction; Conformational states, multiple; Thermodynamic model; Ligand efficacy; Inverse agonist

1. Introduction

Cell-surface receptors linked to effector systems by guanine nucleotide binding (G) proteins represent one of the major cellular mechanisms of transmembrane signaling (Neer, 1995). The G protein-coupled receptors (GPCR) relay information from the exterior to the interior of a cell through a signal transduction (ST) process that involves the formation of a ligand–receptor complex; the formation of the ternary ligand–receptor–G protein complex; the exchange of bound GDP for GTP in the α subunit of the G protein, the dissociation of the α subunit from $\beta\gamma$, and the activation of the effector system. A significant and spectacular advance in the knowledge of the ST process was the relatively recent discovery of constitutively active mutant receptors (see Lefkowitz et al., 1993 and references therein). These receptors are capable of efficiently stimulating G proteins in the absence of the extracellular ligand. These findings suggested that GPCR exists in equilibrium between inactive and active states (Samama et al., 1993; Bond et al., 1995). Two different mechanisms for increasing the population of the active state of the receptor by ligand binding have been suggested: conformational induction and conformational selection (Burgen, 1981; Kenakin, 1995). Conformational induction presupposes that the ligand binds the inactive state of the receptor and then induces the molecular processes that lead to the active state. Conformation selection assumes that unliganded receptors are in equilibrium between the inactive and the active forms and the ligand selectively selects the active conformation of the receptor. However, it is not clear which mechanism is predominant in cellular systems for agonism (Kenakin, 1995, 1996; Bruns, 1996).

Many mathematical models have been put forward for the analysis of concentration–response curves (Katz and Thesleff, 1957; Karlin, 1967; Thron, 1973; DeLean et al., 1980; Black and Leff, 1983; Black and Shankely, 1990; Mackay, 1990;

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Costa et al., 1992; Samama et al., 1993; Bond et al., 1995; Leff, 1995; Weiss et al., 1996). The thermodynamic models taken in those studies include the processes of formation of the ligand–receptor and ligand–receptor–G protein complexes (Black and Leff, 1983; Black and Shankely, 1990; Mackay, 1990); the process of precoupling between the receptor and the G protein (DeLean et al., 1980; Costa et al., 1992); the isomerization step regulating the equilibrium between inactive and active states of the receptor (Katz and Thesleff, 1957; Karlin, 1967; Thron, 1973); the isomerization step regulating the equilibrium between inactive and active states of the receptor both in the absence and in the presence of the ligand (Samama et al., 1993; Bond et al., 1995; Leff, 1995); and the possibility of complex formation between the inactive receptor and the G protein (Weiss et al., 1996). However, none of the above models have quantitatively analyzed the effect of multiple conformational states on the process of receptor activation. The available experimental data from rhodopsin, one of the best experimentally characterized GPCR to date, provides direct evidence for these multiple states between the inactive and the active forms of the receptor (see Lewis and Kliger, 1992 for a review). The structural similarity between rhodopsin and other GPCRs suggests functional similarities, so that multiple equilibria may be a common feature in GPCRs. This hypothesis has recently received experimental support through fluorescent labeling techniques on purified β_2 adrenergic receptors (Gether et al., 1995).

Here we present a generalization of the extended ternary complex (Samama et al., 1993) that incorporates the possibility of multiple conformational states between the inactive and the active forms of the receptor. The main aim of this manuscript is to study the effect of the molecular mechanism of receptor activation, induced by ligand binding, on the process of transmembrane ST. We developed a thermodynamic model of signal transduction within the conformational induction mechanism of receptor activation. The developed mathematical framework can serve in the quantitative analysis of experimental measurements of concentration–response curves, in the precise definition of the widely used concepts of basal activity, potency, maximum response and intrinsic efficacy, and in the definition of agonist, inverse agonist, neutral antagonist and pure antagonist.

2. Methods

2.1. Defining the thermodynamic model

The process of G-protein-mediated transmembrane ST involves steps i to v, as illustrated in Fig. 1 (see the legend). The molecular function of constitutively active receptors (Lefkowitz et al., 1993) and transgenic mice with receptor overexpression (Milano et al., 1994; Bond et al., 1995) provides direct evidence of spontaneous conversion from inactive to the active form of the receptor (iv. *Activation R*) and the formation of the complex between receptors and G protein in the absence of

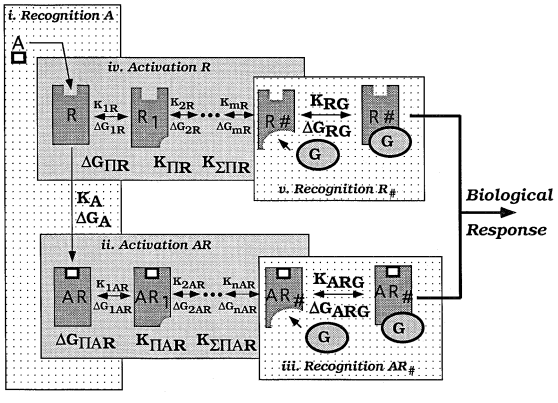

Fig. 1. The process of G protein-mediated transmembrane signal transduction involves steps i to v (see text for details). These processes are characterized by their equilibrium constants defined as: $K_A = [AR]/[A][R]$; $K_{iAR} = [AR_i]/[AR_{i-1}]$; $K_{ARG} = [AR_{\#}G]/[AR_{\#}][G]$; $K_{iR} = [R_i]/[R_{i-1}]$; $K_{RG} = [R_{\#}G]/[R_{\#}][G]$ or by their free energy change given by $\Delta G_i = -RT \ln K_i$. During the algebraic rearrangement of the $[AR_{\#}G]+[R_{\#}G]/[A]$ equations (see text and Appendix A), additional dimensionless constants appeared. They are defined as: $K_{\Pi AR} = \Pi_{i=1}^{n} K_{iAR}$; $K_{\Sigma\Pi AR}^{-1} = \Sigma_{j=1}^{n} \Pi_{i=j}^{n} K_{iAR}^{-1}$; $K_{\Pi R} = \Pi_{i=1}^{m} K_{iR}$; $K_{\Sigma\Pi R}^{-1} = \Sigma_{j=1}^{m} \Pi_{i=j}^{m} K_{iR}^{-1}$. $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ depend on the equilibrium constants of the molecular processes required for receptor activation (K_{iAR}, see ii. *Activation AR*); and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$ depend on the equilibrium constants of the molecular processes required for spontaneous receptor activation in the absence of the ligand (K_{iR}, see iv. *Activation R*). It is important to note that these constants do not include K_A (see i. *Recognition A*), K_{ARG} (see iii. *Recognition AR$_{\#}$*), or K_{RG} (see v. *Recognition R$_{\#}$*). Thus, $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ are constants modeling the equilibrium between inactive AR and active AR$_{\#}$; and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$ are constants modeling the spontaneous conversion from inactive R to the active R$_{\#}$ in the absence of the ligand. $\Delta G_{\Pi R}$ describes the difference in free energy between R$_{\#}$ and R, and $\Delta G_{\Pi AR}$ describes the difference in free energy between AR$_{\#}$ and AR. They are defined as: $\Delta G_{\Pi R} = \Sigma_{i=1}^{m} \Delta G_{iR} = -RT \ln K_{\Pi R}$; $\Delta G_{\Pi AR} = \Sigma_{i=1}^{n} \Delta G_{iAR} = -RT \ln K_{\Pi AR}$.

an agonist (v. *Recognition* $R_{\#}$). It has been suggested that GPCR, as many other proteins, can adopt a number of conformations and that the population of each conformation depends on its energy (see Kenakin, 1996 and references therein). Thus, the unliganded receptor can take up *m* different conformations, R_i, one of them, $R_{\#}$, is able to couple with the G protein. In the absence of the ligand most of the receptor population will be in the inactive state R. There has been some controversy on the conformation of the receptor to which the extracellular ligand binds: conformational induction versus conformational selection (see Kenakin, 1995, 1996; Bruns, 1996) for a discussion). We assume that the extracellular ligand binds the inactive form of the receptor (i. *Recognition A*) and induces the molecular mechanism (ii. *Activation AR*) that leads to the active state (conformational induction). However, the thermodynamic model that we could have developed within the conformational selection mechanism, would have been analogous to the model obtained in this manuscript within the conformational induction mechanism (data not shown). Thus, following ligand binding, the ligand–receptor complex, AR, undergoes rearrangement to one or several intermediates, AR_i, through a series of processes that are transmitted to the cytoplasmatic domains of the receptor (ii. *Activation AR*), facilitating the binding of the ligand-bound receptor to the G-protein. The end state of these molecular processes corresponds to an active state of the receptor (denoted $AR_{\#}$) which is able to couple with the G protein to form the ternary complex $AR_{\#}G$ (iii. *Recognition* $AR_{\#}$). Even though there is the risk of complexity, it is necessary to consider the most general formulation, in which the number of processes required for receptor activation is *n*, as shown in Fig. 1.

2.2. The $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

The molecular processes of transmembrane ST, summarized above, are described by the equations of conservation of receptor ($[R_T]$ is the total concentration of receptor)

$$[R_T] = [R] + [AR] + \sum_{i=1}^{n-1} [AR_i] + [AR_{\#}] + [AR_{\#}G] + \sum_{i=1}^{m-1} [R_i] + [R_{\#}] + [R_{\#}G] \quad (1)$$

and G protein ($[G_T]$ is the total concentration of G protein)

$$[G_T] = [G] + [AR_{\#}G] + [R_{\#}G] \quad (2)$$

Substitution of the equilibrium constants shown in Fig. 1 into all the terms of Eqs. (1) and (2) of conservation of receptor and G protein provides a quadratic relation between $[AR_{\#}G] + [R_{\#}G]$ and [A] (see Appendix A for mathematical details):

$$\left([AR_{\#}G] + [R_{\#}G]\right)^2 - \left([AR_{\#}G] + [R_{\#}G]\right)\left\{[R_T] + [G_T] + \frac{K_A^{-1}K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right)[A]}{K_A^{-1}K_{\Pi R}K_{RG} + K_{\Pi AR}K_{ARG}[A]}\right\} + [R_T][G_T] = 0 \quad (3)$$

The difficulty in manipulating this quadratic equation has been reported for a much simpler thermodynamic model (Black and Shankely, 1990). However, this quadratic equation can be avoided by making two different simplifying assumptions: (a) the concentration of G protein present in a given tissue is smaller than the concentration of receptor: $[G_T] \ll [R_T]$; or (b) the concentration of receptor present in a given tissue is smaller than the concentration of G protein: $[R_T] \ll [G_T]$. These two opposite approaches are characteristics of the analysis of agonist action using the operational model (Black and Leff, 1983; Leff, 1995) and the null method (Mackay, 1990).

(a) *The concentration of G protein in the tissue is smaller than the concentration of receptor* ($[G_T] \ll [R_T]$). Under this assumption the concentrations of receptors bound to G protein are a small part of the total concentration of receptors. In essence this implies that equations of conservation of receptors and G protein take the value of

$$[R_T] = [R] + [AR] + \sum_{i=1}^{n-1} [AR_i] + [AR_{\#}] + \sum_{i=1}^{m-1} [R_i] + [R_{\#}] \times [G_T] = [G] + [AR_{\#}G] + [R_{\#}G] \quad (4)$$

(b) *The concentration of receptor in the tissue is smaller than the concentration of G protein* ($[R_T] \ll [G_T]$). Under this condition the concentration of G protein consumed in forming the ternary complexes ($[AR_{\#}G]$ or $[R_{\#}G]$) is negligible

compared to the concentration of free G protein ([G]). Thus, Eqs. (1) and (2) of conservation of receptor and G protein can be simplified to

$$[R_T] = [R] + [AR] + \sum_{i=1}^{n-1} [AR_i] + [AR_\#] + [AR_\#G] + \sum_{i=1}^{m-1} [R_i] + [R_\#] + [R_\#G] \times [G_T] = [G] \quad (5)$$

Substitution of the equilibrium constants (see legend of Fig. 1) into all the terms of Eq. (4) or Eq. (5) of conservation of receptor and G protein provides the following $[AR_\#G]/[A]$ and $[R_\#G]/[A]$ relationships (see Appendix A for mathematical details):

$$[AR_\#G] = \frac{[G_T][R_T]K_{\prod AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\prod R}\left(1 + K_{\sum\prod R}^{-1}\right) + K_{\prod R}K_{RG}[X_T]\right\} + \left\{K_{\prod AR}\left(1 + K_{\sum\prod AR}^{-1}\right) + K_{\prod AR}K_{ARG}[X_T]\right\}[A]},$$

$$[R_\#G] = \frac{[G_T][R_T]K_A^{-1}K_{\prod R}K_{RG}}{K_A^{-1}\left\{K_{\prod R}\left(1 + K_{\sum\prod R}^{-1}\right) + K_{\prod R}K_{RG}[X_T]\right\} + \left\{K_{\prod AR}\left(1 + K_{\sum\prod AR}^{-1}\right) + K_{\prod AR}K_{ARG}[X_T]\right\}[A]} \quad (6)$$

where $[X_T] = [R_T]$ for $[G_T] \ll [R_T]$; and $[X_T] = [G_T]$ for $[R_T] \ll [G_T]$. The only difference between the $[AR_\#G]/[A]$ and $[R_\#G]/[A]$ relationships, developed from Eq. (4) of conservation of mass ($[G_T] \ll [R_T]$) or from Eq. (5) of conservation of mass ($[R_T] \ll [G_T]$), consists of the presence, in the denominator, of either $[R_T]$ or $[G_T]$, respectively.

Finally, the $[AR_\#G] + [R_\#G]/[A]$ relationship can be calculated as a four parameter function given by:

$$[AR_\#G] + [R_\#G] = \frac{a + b \cdot [A]}{c + d \cdot [A]} \quad (7)$$

in which $a = [G_T][R_T]K_A^{-1}K_{\prod R}K_{RG}$ and $c = K_A^{-1}\{K_{\prod R}(1 + K_{\sum\prod R}^{-1}) + K_{\prod R}K_{RG}[X_T]\}$ are parameters depending of the equilibrium constants of the molecular processes i. *Recognition A*, iv. *Activation R* and v. *Recognition* $R_\#$; and $b = [G_T][R_T]K_{\prod AR}K_{ARG}$ and $d = K_{\prod AR}(1 + K_{\sum\prod AR}^{-1}) + K_{\prod AR}K_{ARG}[X_T]$ are parameters depending of the equilibrium constants of processes ii. *Activation AR* and iii. *Recognition* $AR_\#$.

2.3. Definition of basal activity, maximum response and potency

The basal activity (β) can be defined as the minimum concentration of $[AR_\#G] + [R_\#G]$ and can be obtained as

$$\beta = \lim_{[A]\to 0} [AR_\#G] + [R_\#G] = \frac{[G_T][R_T]K_{RG}}{\left(1 + K_{\sum\prod R}^{-1}\right) + K_{RG}[X_T]} = \frac{a}{c} \quad (8)$$

The obtained value of β (Eq. (8)) properly depends on the equilibrium constants of the molecular processes iv. *Activation R* and v. *Recognition* $R_\#$.

The maximum response (α) is related to the concentration of $AR_\#G + R_\#G$ that can be obtained from an infinite concentration of ligand. It can be calculated, according to the scheme developed by Black and Leff (1983) as:

$$\alpha = \lim_{[A]\to \infty} [AR_\#G] + [R_\#G] = \frac{[G_T][R_T]K_{ARG}}{\left(1 + K_{\sum\prod AR}^{-1}\right) + K_{ARG}[X_T]} = \frac{b}{d} \quad (9)$$

The value of α depends on the equilibrium constants of the molecular processes ii. *Activation AR* and iii. *Recognition* $AR_\#$.

It is important to note that neither α nor β include K_A: the drug-receptor formation constant (i. *Recognition A*). It follows from the above equations that for an efficient transduction of AR into AR# (high values of $K_{\prod AR}$ and $K_{\sum\prod AR}$) or an efficient transduction of R into R# (high values of $K_{\prod R}$ and $K_{\sum\prod R}$); and for an efficient formation of the ternary complex (high values of K_{ARG} or K_{RG}); the first term of the denominator is negligible compared to the last one. Therefore, under the $[G_T] \ll [R_T]$ approach, in which X_T takes the value of R_T, all the G proteins in a given tissue will be complexed with the active form of the receptor, since either $\alpha = [G_T]$ or $\beta = [G_T]$. The value of α or β, in the opposite approach of $[R_T] \ll [G_T]$, is determined by $[R_T]$, which is reasonable due to the deficiency of R_T relative to G_T.

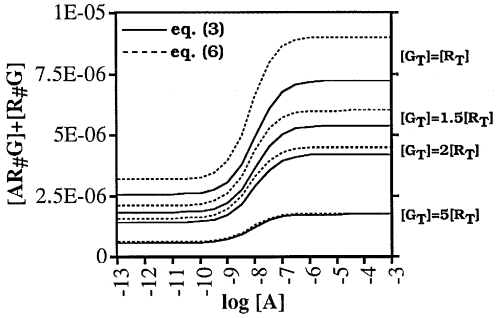

Fig. 2. Computer simulated curves calculated by means of either the general quadratic equation (Eq. (3)) (solid lines) or Eq. (6) for a great excess of G protein compared with receptor in the tissue (dashed lines). The simulations displayed were obtained for different proportions of total concentrations of receptor and G protein in the tissue: $[G_T]=[R_T]=10^{-5}$, $[G_T]=1.5\cdot[R_T]=10^{-5}$, $[G_T]=2\cdot[R_T]=10^{-5}$, and $[G_T]=5\cdot[R_T]=10^{-5}$. The differences obtained between both equations are of a certain magnitude for $[G_T]=[R_T]$ and become negligible for the other simulations. Fixed parameter values of the simulations are defined in Section 2.

The concentration of ligand ($[A_{50}]$) that produces half of the difference in activity between the maximum and the basal, can be calculated from the definition of α (Eq. (9)) and β (Eq. (8)) as:

$$[A_{50}]=\frac{1}{K_A}\cdot\frac{K_{\Pi R}\left(1+K_{\Sigma\Pi R}^{-1}\right)+K_{\Pi R}K_{RG}[X_T]}{K_{\Pi AR}\left(1+K_{\Sigma\Pi AR}^{-1}\right)+K_{\Pi AR}K_{ARG}[X_T]}=\frac{c}{d} \quad (10)$$

In contrast to α and β, $[A_{50}]$ also depends on K_A (i. *Recognition A*), in addition to the equilibrium constants of the molecular processes ii. *Activation AR*, iii. *Recognition* $AR_{\#}$, iv. *Activation R*, and v. *Recognition* $R_{\#}$.

2.4. Computer simulated $[AR_{\#}G]+[R_{\#}G]/[A]$ curves

Fig. 2 shows the $[AR_{\#}G]+[R_{\#}G]/[A]$ curves calculated by means of either the general quadratic equation (Eq. (3)) (solid lines), or Eq. (6) for a vast excess of G protein relative to receptor in the tissue (dashed lines). The simulations displayed were obtained for different proportions of total concentrations of receptor and G protein in the tissue: $[G_T]=[R_T]=10^{-5}$, $[G_T]=1.5\cdot[R_T]=10^{-5}$, $[G_T]=2\cdot[R_T]=10^{-5}$ and $[G_T]=5\cdot[R_T]=10^{-5}$, and default input parameters (see below). In all the cases Eq. (6) predicts higher concentrations of $[AR_{\#}G]+[R_{\#}G]$ than the quadratic equation (Eq. (3)). The differences obtained between both equations are of a certain magnitude for $[G_T]=[R_T]$ and become negligible for the other simulations in which $[G_T]$ is 1.5, 2 and 5 times $[R_T]$. For the β-adrenoceptor response pathway in murine S49 lymphoma cells the following stoichiometry of the ST process has been described: approx. 1500 receptors, 100 000 G_s proteins and $\leq$ 10 000 adenylyl cyclase moieties per cell (Morgan, 1993). If these results can be extrapolated to other systems, the concentration of G_T in the cell is 50–75 times higher than the concentration of R_T ($[R_T]\ll[G_T]$). On the other hand, in over-expressed recombinant systems, there may indeed be the opposite approach, in which the concentration of G_T in the tissue is smaller than the concentration of R_T ($[G_T]\ll[R_T]$). It is important to note that the $[AR_{\#}G]+[R_{\#}G]/[A]$ curves obtained in tissue A with $[G_T^A]\ll[R_T^A]$ would have the same shape as the curve obtained in tissue B with $[R_T^B]\ll[G_T^B]$ due to the complete symmetry of Eq. (6) (if $[G_T^A]=[R_T^B]$ and $[R_T^A]=[G_T^B]$). Thus, for the sake

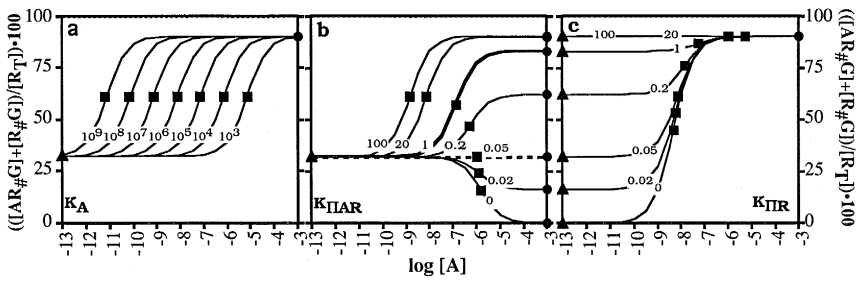

Fig. 3. Computer simulated curves of (a) ligands having different values of association constant K_A for the receptor (i. *Recognition A*); (b) ligand–receptor complexes having different equilibrium constants, $K_{\Pi AR}$, of the molecular processes that lead to the active form of the receptor (ii. *Activation AR*); and (c) receptors having different equilibrium constants, $K_{\Pi R}$, of the molecular processes that lead to the active form of the receptor in the absence of the ligand (iv. *Activation R*). Fixed parameter values of the simulations are defined in Section 2.

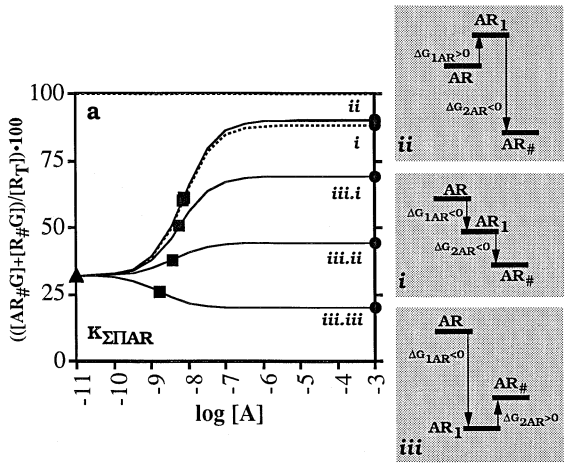

Fig. 4. Influence of the mechanism of receptor activation in the signal transduction process. The mechanism of receptor activation occurs in two molecular steps (see Table 2 for details). Computer simulated curves of the following mechanisms: (i) AR_1 is energetically located between AR and $AR_{\#}$ (broken line); (ii) AR_1 is less stable than AR; and (iii) AR_1 is more stable than $AR_{\#}$. Fixed parameter values of the simulations are defined in Table 2 and in Section 2.

of simplicity, the simulations presented in this manuscript will be carried out by means of Eq. (6) corresponding to only one of these approaches: the $[R_T] \ll [G_T]$.

The computer simulation of model Eq. (6) will be represented as the ratio between $[AR_{\#}G] + [R_{\#}G]$ and $[R_T]$ versus log[A] (Figs. 3 and 4, see below). The basal activity (β, solid triangle), maximum response (α, solid circles), and potency ($[A_{50}]$, solid squares) will be calculated, according to Eqs. (8)–(10) and depicted for each curve. Default input parameters for the simulations are: $K_A = K_{ARG} = K_{RG} = 10^6$, $n = m = 1$, $K_{\Pi AR} = K_{\Sigma\Pi AR} = K_{1AR} = 20$, $K_{\Pi R} = K_{\Sigma\Pi R} = K_{1R} = 0.05$, $[R_T] = 10^{-7}$, $[G_T] = 10^{-5}$. Corresponding to any equilibrium constant, there is a free energy change given by $\Delta G_i = -RT \ln K_i$ where $RT = 0.616$ kcal/mol at 310 K.

3. Results

3.1. Chemical definition of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$, and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$

$K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ are constants modeling the equilibrium between inactive AR and active $AR_{\#}$; and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$ are constants modeling the spontaneous conversion from inactive R to the active $R_{\#}$ in the absence of the ligand (see legend to Fig. 1). The higher the value of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$, the more efficiently the agonist activates the receptor. Similar trends are found for $K_{\Pi R}$ and $K_{\Sigma\Pi R}$ in the spontaneous activation of the receptor. The complexity of these constants depends on the number of processes required for receptor activation. For the simplest activation mechanisms, in which the number of processes required for activation is one ($n = 1$, $m = 1$), these constants take the value of $K_{\Pi AR} = K_{\Sigma\Pi AR} = K_{1AR}$; $K_{\Pi R} = K_{\Sigma\Pi R} = K_{1R}$.

Table 1
Influence of the number of processes required for receptor activation in the presence (n) and in the absence (m) of the ligand, on the equilibrium constants modeling the equilibrium between the inactive and the active forms of the receptor ($K_{\Pi AR}$ or $K_{\Pi R}$ and $K_{\Sigma\Pi AR}$ or $K_{\Sigma\Pi R}$)

m / n	ΔG_{iR} / ΔG_{iAR}	K_{iR} / K_{iAR}	$\Delta G_{\Pi R}$ / $\Delta G_{\Pi AR}$	$K_{\Pi R}$ / $K_{\Pi AR}$	$K_{\Sigma\Pi R}$ / $K_{\Sigma\Pi AR}$
1	−1.845	20.000	−1.845	20.000	20.000
2	−0.923	4.472	−1.845	20.000	3.655
3	−0.615	2.714	−1.845	20.000	1.805
4	−0.461	2.115	−1.845	20.000	1.173
5	−0.369	1.821	−1.845	20.000	0.864
6	−0.308	1.647	−1.845	20.000	0.682
7	−0.264	1.534	−1.845	20.000	0.562

$\Delta G_{\Pi AR}$ or $\Delta G_{\Pi R}$, the free energy difference (kcal/mol) between the inactive and the active form of the receptor; ΔG_{iAR} or ΔG_{iR}, the free energy difference (kcal/mol) between the n or m molecular steps of receptor activation; and K_{iAR} or K_{iR}, the equilibrium constant of the n or m molecular steps of receptor activation.

Table 1 shows the effect of varying the number of processes required for receptor activation in the presence (n) and in the absence (m) of the ligand on $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$, and $K_{\Pi R}$ and $K_{\Sigma\Pi R}$, respectively. The difference in free energy between inactive and active receptor was set as a constant (the value of $\Delta G_{\Pi AR} = \Delta G_{\Pi R} = -1.845$ kcal/mol). The energy difference between inactive and active receptor is overcome by ligand binding through n molecular processes of ΔG_{iAR} kcal/mol, $i = 1, \ldots, n$; or by spontaneous activation of the receptor through m molecular processes of ΔG_{iR} kcal/mol, $i = 1, \ldots, m$. For the sake of simplicity, the free energy difference between the n or m steps required for receptor activation was considered invariable and calculated as the difference in free energy between the inactive and active receptor divided by the number of steps ($\Delta G_{iAR} = \Delta G_{\Pi AR}/n$; $\Delta G_{iR} = \Delta G_{\Pi R}/m$). As can be seen in Table 1, the final values of $K_{\Pi AR}$ and $K_{\Pi R}$ do not depend on the number of processes required for receptor activation. The values of $K_{\Pi AR}$ and $K_{\Pi R}$ remain constant in all simulations. $K_{\Pi AR}$ and $K_{\Pi R}$ are only function of the free energy difference between inactive and active receptor (in these simulations $\Delta G_{\Pi AR} = -1.845$ kcal/mol, $K_{\Pi AR} = 20$; $\Delta G_{\Pi R} = -1.845$ kcal/mol, $K_{\Pi R} = 20$). The situation is different in the analysis of $K_{\Sigma\Pi AR}$ or $K_{\Sigma\Pi R}$. The higher the number of steps necessary for receptor activation, the smaller the values of $K_{\Sigma\Pi AR}$ or $K_{\Sigma\Pi R}$. However, $K_{\Sigma\Pi AR}$ and $K_{\Sigma\Pi R}$ are always comprised in the range $0 < K_{\Sigma\Pi AR} \leq K_{\Pi AR}$ or $0 < K_{\Sigma\Pi R} \leq K_{\Pi R}$. $K_{\Sigma\Pi AR}$ or $K_{\Sigma\Pi R}$ achieve the maximum value ($K_{\Pi AR}$ or $K_{\Pi R}$) for the simplest activation mechanism ($n = 1$ or $m = 1$) and tend to zero for an infinite number of steps. We can conclude that $K_{\Pi AR}$ and $K_{\Pi R}$ are a measure of the energy gap between the inactive and the active form of the receptor in the presence and the absence of the ligand, respectively. On the other hand, $K_{\Sigma\Pi AR}$ is a measure of the chemical mechanism by which the ligand activates the receptor, and $K_{\Sigma\Pi R}$ is a measure of the chemical mechanism by which the receptor is spontaneously activated in the absence of the ligand.

3.2. Effect of i. recognition A on the $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

The computer simulated curves displayed in Fig. 3a correspond to ligands having different values of association constant K_A, and thus different binding affinity for the receptor. An increase in the value of K_A decreases the $[A_{50}]$ of the ligand (solid squares) whereas α (solid circles) and β (solid triangles) remain unaltered. Therefore, α and β are independent of the affinity of the ligand for the receptor. This finding is reasonable since Eq. (9) of α and Eq. (8) of β do not include the drug-receptor formation constant, K_A. The change in $[A_{50}]$ for each ligand in the simulated curves is of the same magnitude as the change in K_A (see Eq. (10)).

3.3. Effect of ii. activation AR and iv. activation R on the $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

The simulations displayed in Fig. 3b correspond to ligand–receptor complexes having different equilibrium constants ($K_{\Pi AR}$) of the molecular processes related to ligand binding and leading to the active form of the receptor. The simulations were carried out for the simplest activation mechanism, in which the number of processes required for receptor activation is only one ($n = 1$, $K_{\Pi AR} = K_{\Sigma\Pi AR} = K_{1AR}$). Clearly, the higher the value of $K_{\Pi AR} = K_{\Sigma\Pi AR}$ the more efficiently a ligand propitiates the maximum concentration of $AR_{\#}G$ and the higher the value of α (solid circles) is. The basal activity, β, remains unaltered with the process of ii. *Activation AR*.

The effect of varying the equilibrium constants modeling the spontaneous conversion from inactive R to the active $R_{\#}$ in the absence of the ligand ($K_{\Pi R}$) is shown in Fig. 3c. All the simulations were carried out for the simplest activation mechanism ($m = 1$, $K_{\Pi R} = K_{\Sigma\Pi R} = K_{1R}$). An increase in the value of $K_{\Pi R} = K_{\Sigma\Pi R}$ increases the basal activity, β (solid triangles), whereas the efficacy, α (solid circles) remains unaltered. The values of $K_{\Pi R}$ chosen for the simulations are depicted in Fig. 3c. The coincidence of these values to those employed in Fig. 3b for $K_{\Pi AR}$, leads to a total correspondence between the maximum response obtained in Fig. 3b and the basal activity obtained in Fig. 3c.

3.3.1. Definition of agonists, inverse agonists and antagonists

Depending on the relative value of α and β the extracellular ligands can be classified as: (a) ligands that trigger the maximum value of α in a given tissue (full agonists), (b) ligands that trigger a value of α comprised between β and the maximum value of α (partial agonists), (c) ligands that do not alter the response of the system ($\alpha = \beta$, neutral antagonists), (d) ligands with α values lower than β (inverse agonists (Bond et al., 1995)) and (e) ligands with α equal to 0 (pure antagonists). It would be very valuable to define a cutoff values of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ that determines the pharmacological behavior of the ligand. Fig. 3b shows, in bold, the computer simulated curve for $K_{\Pi AR} = K_{\Sigma\Pi AR} = 1$ in which the ligand has no preference between the inactive and active forms of the receptor. Although the ligand is not efficient in generating $AR_{\#}$, high values of α are obtained. The magnitude of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ required for obtaining the maximum value of α (full agonists) depends on the equilibrium constants of the other molecular processes that form the ST system (the formation of the ternary complex, the exchange of bound GDP for GTP, the activation of the effector system, and so on). Thus, very

efficient ST systems will require ligands with moderate values of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ whereas less efficient ST systems will require ligands with higher values, for obtaining identical values of α.

The situation is different in the definition of neutral antagonists and thus in the discrimination between partial and inverse agonists. Because the relative values of α and β define the pharmacological behavior of these ligands, it seems logical to calculate the value of $K_{\Pi AR}$ and $K_{\Sigma\Pi AR}$ that reproduces equal values of α and β. Thus, this condition (see broken line in Fig. 3b, and Eqs. (8) and (9) is:

$$\frac{[G_T][R_T]K_{RG}}{\left(1+K_{\Sigma\Pi R}^{-1}\right)+K_{RG}[X_T]}=\frac{[G_T][R_T]K_{ARG}}{\left(1+K_{\Sigma\Pi AR}^{-1}\right)+K_{ARG}[X_T]} \tag{11a}$$

or

$$K_{ARG}^{-1}\left(1+K_{\Sigma\Pi AR}^{-1}\right)=K_{RG}^{-1}\left(1+K_{\Sigma\Pi R}^{-1}\right) \tag{11b}$$

The above equation can be easily simplified to $K_{\Sigma\Pi AR}=K_{\Sigma\Pi R}$ (if $K_{ARG}=K_{RG}$), in which the competition between the chemical mechanism of receptor activation by ligand binding (monitored by $K_{\Sigma\Pi AR}$) and the chemical mechanism of spontaneous receptor activation in the absence of the ligand (monitored by $K_{\Sigma\Pi R}$) determines the pharmacological behavior of these ligands. It is important to note that the equilibrium constants that measure the difference in energy between inactive and active receptor, $K_{\Pi AR}$ and $K_{\Pi R}$, do not define the pharmacological behavior of these extracellular ligands (see below). Thus, ligands that activate the receptor through values of $K_{\Sigma\Pi AR}>K_{\Sigma\Pi R}$ (or $K_{\Sigma\Pi AR}/K_{\Sigma\Pi R}>1$) will behave as either full or partial agonist, ligands that activate the receptors through values of $K_{\Sigma\Pi AR}=K_{\Sigma\Pi R}$ (or $K_{\Sigma\Pi AR}/K_{\Sigma\Pi R}=1$) will not alter the response and will behave as neutral antagonists, whereas ligands that activate the receptor through values of $K_{\Sigma\Pi AR}$ in the range $K_{\Sigma\Pi R}>K_{\Sigma\Pi AR}>0$ (or $1>K_{\Sigma\Pi AR}/K_{\Sigma\Pi R}>0$) will behave as inverse agonists. Finally, ligands that cannot activate the receptor ($n=0$, $K_{\Sigma\Pi AR}=0$), do not produce intracellular [$AR_{\#}G$] ($\alpha=0$) and act as receptor blockers (pure antagonists).

3.3.2. Influence of the chemical mechanism of receptor activation in the ST process and in the definition of the pharmacological behavior of ligands

To further illustrate how the mechanism of receptor activation influences α, Fig. 4 shows the computer simulation curves in which the energy difference between AR and $AR_{\#}$ (monitored by $\Delta G_{\Pi AR}$ and $K_{\Pi AR}$) was set constant, but the chemical mechanism of receptor activation (monitored by $K_{\Sigma\Pi AR}$) was systematically varied (see Scheme in Fig. 4). Thus, for an extracellular ligand that drives the energy gap between AR and $AR_{\#}$ (i.e., $\Delta G_{\Pi AR}=-1.84$ kcal/mol, $K_{\Pi AR}=20$) in two activation steps, we aim to compare the following chemical mechanisms: (i) AR_1 is energetically located between AR and $AR_{\#}$ (both processes are exoergic with $\Delta G_{1AR}=\Delta G_{2AR}=-0.92$ kcal/mol), see broken line in Fig. 4, Scheme *i* in Fig. 4 and Set *i* in Table 2; (ii) the AR_1 is less stable than AR (the first step is endoergic, $\Delta G_{1AR}>0$; and the second exoergic, $\Delta G_{2AR}<0$), see *ii* in Fig. 4 and Set *ii* in Table 2; and (iii) AR_1 is more stable than $AR_{\#}$ (the first step is exoergic, $\Delta G_{2AR}<0$, and the second endoergic, $\Delta G_{2AR}>0$), see *iii* in Fig. 4 and Set *iii* in Table 2. Analysis of the data shown in Table 2 and Fig. 4 indicates that there is a clear relationship between the value of $K_{\Sigma\Pi AR}$ and α. The closer the value of $K_{\Sigma\Pi AR}$ is to its maximum value ($K_{\Pi AR}$), the higher the value of α. Furthermore, the computer-simulated curves denoted as Set *ii* (AR_1 is less stable than AR) reproduces higher values of $K_{\Sigma\Pi AR}$ and α (more efficient activation mechanism) than both Set *i* and *iii* simulations. We can conclude that unfavorable activation processes might occur. Thus, the extracellular ligand can bring the receptor to an energetically unstable state from which it is energetically favorable to reach $AR_{\#}$. It has been suggested that the process of receptor activation involves a conformational change in the tertiary structure of the ligand–receptor complex, assisted by the conserved Pro residues in the middle of the transmembrane helices

Table 2
Influence of the chemical mechanism of receptor activation in the presence of the ligand ($K_{\Sigma\Pi AR}$) on the maximum response (α) and log[A_{50}]

	n	ΔG_{1AR}	ΔG_{2AR}	K_{1AR}	K_{2AR}	$K_{\Sigma\Pi AR}$	α	log[A_{50}]
i	2	−0.92	−0.92	4.47	4.47	3.65	88.7	−8.2
ii.i	2	0.75	−2.60	0.30	67.58	15.43	90.4	−8.2
ii.ii	2	1.50	−3.35	0.09	228.33	18.39	90.5	−8.2
ii.iii	2	2.25	−4.10	0.03	771.50	19.50	90.5	−8.2
iii.i	2	−2.60	0.75	67.58	0.30	0.30	69.3	−8.3
iii.ii	2	−3.35	1.50	228.33	0.09	0.09	44.5	−8.5
iii.iii	2	−4.10	2.25	771.50	0.03	0.03	20.2	−8.8

$m=1$, $\Delta G_{\Pi R}=1.845$ kcal/mol, $K_{\Pi R}=K_{\Sigma\Pi R}=0.05$, $\Delta G_{\Pi AR}=-1.845$ kcal/mol, $K_{\Sigma\Pi AR}=20.0$.

(Zhang and Weinstein, 1993). The importance of these Pro residues in the ST process has received experimental support through site directed mutagenesis (Wess et al., 1993). Therefore, the ligand might produce unfavorable changes in the receptor binding site that triggers the significant change in the conformational properties of the receptors that are transmitted to the intracellular site.

Another significant issue that can be analyzed with the simulations shown in Table 2 and Fig. 4 is the importance of the chemical mechanism of receptor activation ($K_{\Sigma\Pi AR}$ and $K_{\Sigma\Pi R}$) rather than the difference in energy between both states of the receptor ($K_{\Pi AR}$ and $K_{\Pi R}$) in the definition of the pharmacological behavior of the extracellular ligands (this is only relevant for activation mechanisms with more than one molecular step). The default input parameters for the simulations shown in Table 2 and Fig. 4 are: $m = 1$, $\Delta G_{\Pi R} = 1.845$ kcal/mol, $K_{\Pi R} = K_{\Sigma\Pi R} = 0.05$, $n = 2$, $\Delta G_{\Pi AR} = -1.845$ kcal/mol, $K_{\Pi R} = 20$. Thus, in all shown simulations, $K_{\Pi AR}$ is higher than $K_{\Pi R}$. However, a complete spectrum of activities, ranging from full to inverse agonist, are obtained. It is noteworthy that one of these simulations (see *iii.iii* in Table 2) reproduces a very inefficient activation mechanism ($K_{\Sigma\Pi AR} = 0.026 < K_{\Sigma\Pi R} = 0.05$), causing the ligand to behave as an inverse agonist (see Fig. 4).

The extended ternary model of agonist action (Samama et al., 1993; Bond et al., 1995; Leff, 1995) suggests that efficacy is related to the ratio of affinities for the inactive and active forms of the receptor. Ligands that bind the receptor with higher affinity for the active ($K_A^{\#}$) than the inactive (K_A) form are denominated agonists ($K_A^{\#}/K_A > 1$), whereas ligands with opposite preference for the forms of the receptor are denominated inverse agonists ($K_A^{\#}/K_A < 1$). It can be shown that this ratio of affinities is always equal to the ratio of equilibrium constants modeling the difference in energy between inactive and active form of the receptor ($K_A^{\#}/K_A = K_{\Pi AR}/K_{\Pi R}$). Moreover, in the particular case of the simplest activation mechanism (only two states of the receptor exist), the ratio of affinities is equal to the ratio of equilibrium constants modeling the chemical mechanism of receptor activation ($K_A^{\#}/K_A = K_{\Pi AR}/K_{\Pi R} = K_{\Sigma\Pi AR}/K_{\Sigma\Pi R}$). Thus, the thermodynamic model developed in this work, that incorporates the possibility of multiple conformational states between the inactive and the active forms of the receptor, provides the generalization of the extended ternary model and a more general definition of the pharmacological behavior of the extracellular ligand.

The only processes of the ST pathway (see Fig. 1) that are directly influenced by the extracellular ligand are: (i) the recognition of the ligand by the receptor, and (ii) the activation of the receptor by ligand binding. Therefore, the maximum concentration of $AR_{\#}G$ obtained in a given tissue, only depends on the process of receptor activation (ii: *Activation AR*), since the affinity of the ligand to the receptor (i: *Recognition A*) does not affect α (see above and Eq. (9)). Thus, intrinsic efficacy of the agonist can be defined as the molecular processes induced by ligands in the receptor that lead to the active form of the receptor, $AR_{\#}$. The main conclusion of this work is that both the energetics (monitored by $K_{\Pi AR}$) and the chemical mechanism (monitored by $K_{\Sigma\Pi AR}$) of the processes of receptor activation are important in eliciting the biological response.

3.4. Effect of iii. Recognition $AR_{\#}$ and v. Recognition $R_{\#}$ on the $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

The effect of varying K_{ARG} on the intracellular concentration of $[AR_{\#}G] + [R_{\#}G]$ is shown in Fig. 5a. The line in bold was obtained using default input parameters (see above), whereas the other computer simulated curves represent the effect of increasing or decreasing the default free energy of binding between $AR_{\#}$ and the G protein (ΔG_{ARG}) by values ranging from -3 to $+3$ kcal/mol. It is clear that the higher the value of K_{ARG}, the higher the value of α is. However, the change in α is not of the same magnitude whether ΔG_{ARG} increases or decreases. An increase of $+3$ kcal/mol in the value of ΔG_{ARG} increases α from 90.5% to 99.5%, whereas a decrease of -3 kcal/mol in ΔG_{ARG} produces a more significant

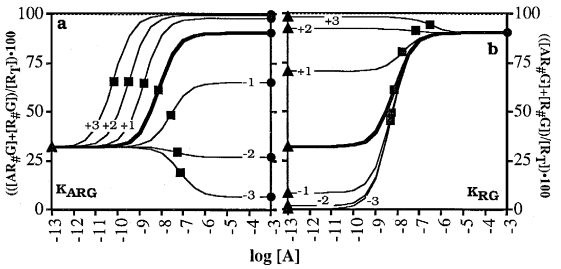

Fig. 5. Computer simulated curves of (a) ligand-bound, activated receptor complexes having different values of association constant, K_{ARG}, for the G protein (iii. *Recognition* $AR_{\#}$); and (b) activated receptor having different association constant, K_{RG}, for the G protein in the absence of the ligand (v. *Recognition* $R_{\#}$). The lines in bold were obtained using default input parameters (see Section 2), whereas the other computer simulated curves represent the effect of increasing or decreasing the default free energy of binding between (ΔG_{ARG} or ΔG_{RG}) by values ranging from -3 to $+3$ kcal/mol.

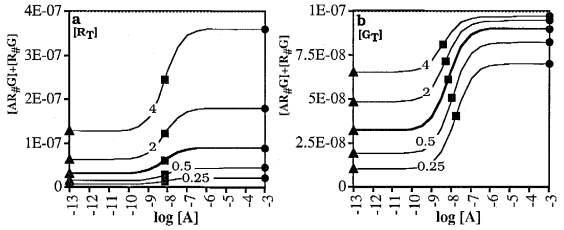

Fig. 6. Computer simulated curves in which (a) the total concentration of receptor $[R_T]$, and (b) the total concentration of G protein $[G_T]$ are multiplied by factors of 0.25, 0.5, 2, and 4. The line in bold was obtained using default input parameters (see Section 2).

change of α from 90.5% to 6.8%. Fig. 5b shows the effect of varying ΔG_{RG}, the free energy of binding between $R_{\#}$ and the G protein in the absence of the ligand, on the intracellular concentration of $[AR_{\#}G] + [R_{\#}G]$. As expected, the higher the value of K_{RG}, the higher the value of β is. In contrast to the observed variation of α with ΔG_{ARG}, an increase of $+3$ kcal/mol in the value of ΔG_{RG} produces a very significant increases of β from 32.6% to 98.4%, whereas a decrease of -3 kcal/mol in ΔG_{RG} decreases β only from 32.6% to 0.4%.

There is a clear relationship between these changes and the magnitude of the activation process. The lower the efficiency of either ii. *Activation AR* or iv. *Activation R* is (lower values of $K_{\Sigma\Pi AR}$ or $K_{\Sigma\Pi R}$), the more noticeable the effect of increasing the binding between the receptor and the G protein (higher values of K_{ARG} or K_{RG}) is on either α or β. A powerful technique to study ST mechanisms is the genetic expression. Mutations in different parts of the GPCR can increase their constitutive activity achieving various levels of basal activity (Kjelsberg et al., 1992; Ren et al., 1993; Rhee and Jacobson, 1996; Scheer et al., 1996). Most of these mutations are located on or near the intracellular domains of the receptor that form the binding site for the G protein. Thus, if these mutations increase K_{RG} and K_{ARG} in identical manner, without changing the efficiency of ii. *Activation AR* or iv. *Activation R* (it seems reasonable to assume that $K_{\Sigma\Pi R} \ll K_{\Sigma\Pi AR}$), the increase in basal activity will be much higher than in maximum response, causing the mutant receptor to be constitutively active. Thus, special attention should be taken in the analysis and interpretation of the molecular mechanism of constitutively active mutant receptors. Similarly, the effect of K_{ARG} in the maximum response (α) will depend on the pharmacological behavior of the ligand. The extracellular ligand can be classified as full agonist, partial agonist, neutral antagonist and inverse agonist, from higher to lower value of $K_{\Sigma\Pi AR}$ (see above). Thus, a similar increase in K_{ARG} will be more noticeable in ligands with lower values of $K_{\Sigma\Pi AR}$ (inverse agonist) than with higher values of $K_{\Sigma\Pi AR}$ (full agonists).

3.5. Effect of tissue components on the $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

Recent experiments in transgenic mice overexpressing the β_2-adrenergic receptor have shown that both basal and isoproterenol-stimulated cyclase activity were increased in these animals relative to controls (Milano et al., 1994). Thus, there must be two components in the magnitude of intracellular concentration of $[AR_{\#}G] + [R_{\#}G]$, namely, a thermodynamic component, which is a function of the equilibrium constants of the processes connecting receptor occupancy to the formation of $[AR_{\#}G] + [R_{\#}G]$; and a tissue component, which is a function of $[R_T]$ and $[G_T]$. The effect of the first component has already been explored in the previous sections. We present below the effect of the tissue component on the intracellular concentration of $[AR_{\#}G] + [R_{\#}G]$.

The simulations displayed in Fig. 6 show the $[AR_{\#}G] + [R_{\#}G]/[A]$ curves obtained for different total concentrations of receptor, and G protein in the tissue. In these simulations the Y axis corresponds to $[AR_{\#}G] + [R_{\#}G]$ instead of the ratio between $[AR_{\#}G] + [R_{\#}G]$ and $[R_T]$. The line in bold was obtained using default input parameters (see above), whereas the other computer simulated curves represent the effect of multiplying $[R_T]$ (Fig. 6a), or $[G_T]$ (Fig. 6b) by factors of 0.25, 0.5, 2 and 4. It seems clear that, in all the cases, the higher the value of $[R_T]$ or $[G_T]$; the larger the values of α and β are. However, the observed changes in α and β with the variation of the tissue components are not of the same magnitude for $[R_T]$ or $[G_T]$. As the number of receptors, $[R_T]$, changes by a given factor (0.25, 0.5, 2 or 4, see Fig. 6a), the values of α and β change by exactly the same factor (0.25, 0.5, 2, or 4, respectively), whereas $[A_{50}]$ remains unaltered. This finding is reasonable because Eqs. (8) and (9) of β and α are proportional to $[R_T]$, and Eq. (10) of $[A_{50}]$ is independent of $[R_T]$, under the $[R_T] \ll [G_T]$ approach. The effect of $[G_T]$ on the ST process is not as noticeable as for $[R_T]$. The magnitude of β changes by factors of 0.33, 0.60, 1.51 or 2.03; and the magnitude of α by factors of 0.78, 0.91, 1.05 or 1.08; if $[G_T]$ is changed. The importance of $[R_T]$ relative to $[G_T]$ in the ST process is reasonable because the simulations were obtained on the assumption that $[R_T] < [G_T]$, so that R_T is the determinant species in the formation of the receptor-G protein complex. Computer simulation curves obtained according to the $[G_T] \ll [R_T]$ approach would have reached the opposite conclusion

that $[G_T]$ is more important than $[R_T]$ in the ST process. Therefore, we can conclude that the process of ST is very sensitive to the variation of the total concentration of the less abundant species (either R_T or G_T) in the tissue.

4. Conclusions

The present study supplies, at the thermodynamic level, the analytical equations for the understanding and interpretation of the ST process. Specifically, the thermodynamic model incorporates the possibility of multiple conformational states between the inactive and the active forms of the receptor. It provides the generalization of the extended ternary model (Samama et al., 1993; Bond et al., 1995; Leff, 1995) and a more general definition of the pharmacological behavior of the extracellular ligand. Moreover, these equations can be applied to the analysis of concentration–response curves obtained in biochemical, in molecular biology, and in pharmacological experiments. Thus, the changes in concentration–response curves obtained for a set of chemically different ligands; or for wild, mutant, chimeric, or constitutively active receptors; or for different concentrations of R_T, or G_T, among many other applications, can be analyzed in a mechanistic context. Furthermore, it provides the link between the macroscopic behavior, expressed in the concentration–response curves, and the microscopic behavior, expressed in the energetics of the molecular mechanisms of ST.

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Appendix A

A.1. General quadratic equation of the $[AR_{\#}G] + [R_{\#}G] / [A]$ relationship

The process of G protein-mediated transmembrane signal transduction involves steps i to v (see the text and Fig. 1 for details). The formation of the complex between receptor and G protein, both in the absence ($R_{\#}G$) and in the presence ($AR_{\#}G$) of the ligand, are considered in the present model as the active species which promote the cascade of events that finally lead to the formation of the second messengers. Thus, we aim to express the concentration of all the species intervening in the equations of conservation of receptor and G protein

$$[R_T] = [R] + [AR] + \sum_{i=1}^{n-1} [AR_i] + [AR_{\#}] + [AR_{\#}G] + \sum_{i=1}^{m-1} [R_i] + [R_{\#}] + [R_{\#}G] \quad (A.1)$$

$$[G_T] = [G] + [AR_{\#}G] + [R_{\#}G] \quad (A.2)$$

as a function of either $[R_{\#}G]$ or $[AR_{\#}G]$. Mathematical manipulation of the equilibrium constants describing steps i to v:

$$K_A = \frac{[AR]}{[A][R]}; \qquad K_{iAR} = \frac{[AR_i]}{[AR_{i-1}]}; \qquad K_{ARG} = \frac{[AR_{\#}G]}{[AR_{\#}][G]}; \qquad K_{iR} = \frac{[R_i]}{[R_{i-1}]}; \qquad K_{RG} = \frac{[R_{\#}G]}{[R_{\#}][G]} \quad (A.3)$$

gives us the following relations between species:

$$[AR] = \frac{[AR_{\#}G]}{\prod_{i=1}^{n} K_{iAR} K_{ARG} [G]}, \qquad [R] = \frac{[R_{\#}G]}{\prod_{i=1}^{m} K_{iR} K_{RG} [G]} \quad (A.4)$$

$$[AR_i] = \frac{[AR_{\#}G]}{\prod_{j=i+1}^{n} K_{jAR} K_{ARG} [G]}, \qquad [R_i] = \frac{[R_{\#}G]}{\prod_{j=i+1}^{m} K_{jR} K_{RG} [G]} \quad (A.5)$$

$$[AR_{\#}] = \frac{[AR_{\#}G]}{K_{ARG}[G]}, \qquad [R_{\#}] = \frac{[R_{\#}G]}{K_{RG}[G]} \quad (A.6)$$

$$[AR_{\#}G] = [R_{\#}G] \cdot \frac{\prod_{i=1}^{n} K_{iAR}}{\prod_{i=1}^{m} K_{iR}} \cdot \frac{K_{ARG}}{K_{RG}} \cdot K_A [A] \quad (A.7)$$

Insertion of Eqs. (A.4), (A.5) and (A.6) into Eq. (A.1) of conservation of receptors gives:

$$[R_T] = [R_{\#}G]\left\{1 + \frac{1}{K_{RG}[G]}\left(1 + \prod_{i=1}^{m} K_{iR}^{-1} + \sum_{i=1}^{m-1}\prod_{j=i+1}^{m} K_{jR}^{-1}\right)\right\} + [AR_{\#}G]\left\{1 + \frac{1}{K_{ARG}[G]}\left(1 + \prod_{i=1}^{m} K_{iAR}^{-1} + \sum_{i=1}^{m-1}\prod_{j=i+1}^{m} K_{jAR}^{-1}\right)\right\} \quad (A.8)$$

which let us to define the following dimensionless constant (see the text for the chemical definition of these constants):

$$K_{\Pi R} = \prod_{i=1}^{m} K_{iR}; \qquad K_{\Sigma\Pi R}^{-1} = \sum_{i=1}^{m}\prod_{j=i}^{m} K_{jR}^{-1} = \prod_{i=1}^{m} K_{iR}^{-1} + \sum_{i=1}^{m-1}\prod_{j=i+1}^{m} K_{jR}^{-1}$$

$$K_{\Pi AR} = \prod_{i=1}^{n} K_{iAR}; \qquad K_{\Sigma\Pi AR}^{-1} = \sum_{i=1}^{n}\prod_{j=i}^{n} K_{jAR}^{-1} = \prod_{i=1}^{n} K_{iAR}^{-1} + \sum_{i=1}^{n-1}\prod_{j=i+1}^{n} K_{jAR}^{-1} \quad (A.9)$$

Eq. (A.7) is substituted into the $[AR_{\#}G]$ term of Eq. (A.8), in order to obtain the desirable $[R_{\#}G]/[A]$ relationship:

$$[R_{\#}G] = \frac{[G][R_T]K_A^{-1}K_{\Pi R}K_{RG}}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi R}K_{RG}[G]\right\} + \left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right) + K_{\Pi AR}K_{ARG}[G]\right\}[A]} \quad (A.10)$$

Analogously, substitution of Eq. (A.7) into the $[R_{\#}G]$ term of Eq. (A.8) provides the $[AR_{\#}G]/[A]$ relationship:

$$[AR_{\#}G] = \frac{[G][R_T]K_{\Pi AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi R}K_{RG}[G]\right\} + \left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right) + K_{\Pi AR}K_{ARG}[G]\right\}[A]} \quad (A.11)$$

Addition of Eqs. (A.10) and (A.11) provides the $[AR_{\#}G] + [R_{\#}G]/[A]$ relationship:

$$[AR_{\#}G] + [R_{\#}G] = \frac{[G][R_T]K_A^{-1}K_{\Pi R}K_{RG} + [G][R_T]K_{\Pi AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi R}K_{RG}[G]\right\} + \left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right) + K_{\Pi AR}K_{ARG}\right\}[A]} \quad (A.12)$$

The concentration of $[AR_{\#}G] + [R_{\#}G]$ in Eq. (A.12) is a non-hyperbolic function of [A] since [G] is not constant. Eq. (A.2) of conservation of G protein can be used to express [G] in terms of $[G_T]$, $[AR_{\#}G]$ and $[R_{\#}G]$:

$$[G] = [G_T] - ([AR_{\#}G] + [R_{\#}G]) \quad (A.13)$$

Substitution of Eq. (A.13) into Eq. (A.12) provides $[AR_{\#}G] + [R_{\#}G]$ in terms of [A]. Note that [G] is also a function of $[AR_{\#}G] + [R_{\#}G]$, so the values of $[AR_{\#}G] + [R_{\#}G]$ at different [A] must be obtained by solving the quadratic equation

$$([AR_{\#}G] + [R_{\#}G])^2 - \left([AR_{\#}G] + [R_{\#}G]\left\{[R_T] + [G_T] + \frac{K_A^{-1}K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right)[A]}{K_A^{-1}K_{\Pi R}K_{RG} + K_{\Pi AR}K_{ARG}[A]}\right\}\right) + [R_T][G_T] = 0 \quad (A.14)$$

A.2. The concentration of receptor in the tissue is smaller than the concentration of G protein ($[R_T] \ll [G_T]$)

Under this conditions $[AR_{\#}G]$ and $[R_{\#}G]$ are negligible relative to [G], so that Eq. (A.13) can be simplified to

$$[G] = [G_T] \quad (A.15)$$

In essence this implies that the relation between $[AR_{\#}G] + [R_{\#}G]$ and [A], previously obtained in Eq. (A.12), follows a hyperbolic function since [G] is a constant. Eq. (A.12) can be rewritten as

$$[AR_{\#}G] + [R_{\#}G] = \frac{[G_T][R_T]K_A^{-1}K_{\Pi R}K_{RG} + [G_T][R_T]K_{\Pi AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right) + K_{\Pi R}K_{RG}[G_T]\right\} + \left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right) + K_{\Pi AR}K_{ARG}[G_T]\right\}[A]} \quad (A.16)$$

A.3. The concentration of G protein in the tissue is smaller than the concentration of receptor ($[G_T] \ll [R_T]$)

Under this assumption Eq. (A.1) of conservation of receptors can be simplified to

$$[R_T] = [R] + [AR] + \sum_{i=1}^{n-1} [AR_i] + [AR_\#] + \sum_{i=1}^{m-1} [R_i] + [R_\#] \tag{A.17}$$

A.3.1. The $[AR_\#]$ / $[A]$ and $[R_\#]$ / $[A]$ relationships

Here, we aim to express the concentration of all the species intervening in Eq. (A.17) of conservation of receptors as a function of $[R_\#]$ or $[AR_\#]$.

$$[AR] = \frac{[AR_\#]}{K_{\Pi AR}}, \qquad [R] = \frac{[R_\#]}{K_{\Pi R}} \tag{A.18}$$

$$[AR_i] = \frac{[AR_\#]}{\prod_{j=i+1}^{n} K_{jAR}}, \qquad [R_i] = \frac{[R_\#]}{\prod_{j=i+1}^{m} K_{jR}} \tag{A.19}$$

$$[AR_\#] = [R_\#] \cdot \frac{K_{\Pi AR}}{K_{\Pi R}} \cdot K_A[A] \tag{A.20}$$

Insertion of Eqs. (A.18) and (A.19) into Eq. (A.17) of conservation of receptors gives:

$$[R_T] = [R_\#]\left\{1 + K_{\Sigma\Pi R}^{-1}\right\} + [AR_\#]\left\{1 + K_{\Sigma\Pi AR}^{-1}\right\} \tag{A.21}$$

Similarly to the process described above, substitution to Eq. (A.20) to the $[R_\#]$ and $[AR_\#]$ terms of Eq. (A.21) provides the $[AR_\#]/[A]$ and $[R_\#]/[A]$ relationships:

$$[R_\#] = \frac{[R_T] K_A^{-1} K_{\Pi R}}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right)\right\} + \left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right)\right\}[A]} \tag{A.22}$$

$$[AR_\#] = \frac{[R_T] K_{\Pi AR}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1 + K_{\Sigma\Pi R}^{-1}\right)\right\}\left\{K_{\Pi AR}\left(1 + K_{\Sigma\Pi AR}^{-1}\right)\right\}[A]} \tag{A.23}$$

A.3.2. The $[AR_\#G]$ / $[AR_\#]$ and $[R_\#G]$ / $[R_\#]$ relationships

Substitution of the equilibrium constants K_{ARG} and K_{RG}, defined in Eq. (A.3), into Eq. (A.2) provides the equation of conservation of G protein as a function of $[R_\#G]$ or $[AR_\#G]$:

$$[G_T] = \frac{[R_\#G]}{K_{RG}[R_\#]} + [R_\#G] \cdot \frac{K_{ARG}}{K_{RG}} \cdot \frac{[AR_\#]}{[R_\#]} + [R_\#G] \tag{A.24}$$

$$[G_T] = \frac{[AR_\#G]}{K_{ARG}[AR_\#]} + [AR_\#G] + [AR_\#G] \cdot \frac{K_{RG}}{K_{ARG}} \cdot \frac{[R_\#]}{[AR_\#]} \tag{A.25}$$

From Eqs. (A.24) and (A.25) we can obtained the $[R_\#G]/[R_\#]$ and $[AR_\#G]/[AR_\#]$ relationships:

$$[R_\#G] = \frac{[G_T] K_{RG}[R_\#]}{1 + K_{RG}[R_\#] + K_{ARG}[AR_\#]} \tag{A.26}$$

$$[AR_\#G] = \frac{[G_T] K_{ARG}[AR_\#]}{1 + K_{RG}[R_\#] + K_{ARG}[AR_\#]} \tag{A.27}$$

A.3.3. The $[AR_\#G] + [R_\#G]$ / $[A]$ relationship

The mechanism of signal transduction has been split into two distinguishable mechanisms: activation of the receptor (the $[AR_\#]/[A]$ and $[R_\#]/[A]$ relationships), and formation of the complex with the G protein (the $[AR_\#G]/[AR_\#]$ and

$[R_{\#}G]/[R_{\#}]$ relationships). Insertion of the hyperbolic $[AR_{\#}]/[A]$ and $[R_{\#}]/[A]$ relations into the $[R_{\#}G]/[R_{\#}]$ relation gives the $[R_{\#}G]/[A]$ relationship:

$$[R_{\#}G] = \frac{[G_T][R_T]K_A^{-1}K_{\Pi R}K_{RG}}{K_A^{-1}\left\{K_{\Pi R}\left(1+K_{\Sigma\Pi R}^{-1}\right)+K_{\Pi R}K_{RG}[R_T]\right\}+\left\{K_{\Pi AR}\left(1+K_{\Sigma\Pi AR}^{-1}\right)+K_{\Pi AR}K_{ARG}[R_T]\right\}[A]} \quad (A.28)$$

Analogously, insertion of the hyperbolic $[AR_{\#}]/[A]$ and $[R_{\#}]/[A]$ relations into the $[AR_{\#}G]/[AR_{\#}]$ relation gives the $[AR_{\#}G]/[A]$ relationship

$$[AR_{\#}G] = \frac{[G_T][R_T]K_{\Pi AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1+K_{\Sigma\Pi R}^{-1}\right)+K_{\Pi R}K_{RG}[R_T]\right\}+\left\{K_{\Pi AR}\left(1+K_{\Sigma\Pi AR}^{-1}\right)+K_{\Pi AR}K_{ARG}[R_T]\right\}[A]} \quad (A.29)$$

Addition of Eqs. (A.10) and (A.11) provides the $[AR_{\#}G]+[R_{\#}G]/[A]$ relationship:

$$[AR_{\#}G]+[R_{\#}G] = \frac{[G_T][R_T]K_A^{-1}K_{\Pi R}K_{RG}+[G_T][R_T]K_{\Pi AR}K_{ARG}[A]}{K_A^{-1}\left\{K_{\Pi R}\left(1+K_{\Sigma\Pi R}^{-1}\right)+K_{\Pi R}K_{RG}[R_T]\right\}+\left\{K_{\Pi AR}\left(1+K_{\Sigma\Pi AR}^{-1}\right)+K_{\Pi AR}K_{ARG}[R_T]\right\}[A]} \quad (A.30)$$

This equation has the same form as the equation obtained for the reverse situation, in which the concentration of receptor in the tissue is smaller than the concentration of G protein ($[R_T] \ll [G_T]$). The only difference between the two forms of the $[AR_{\#}G]+[R_{\#}G]/[A]$ relationship consists in the presence, at the denominator, of either $[R_T]$ or $[G_T]$, depending on the $[G_T] \ll [R_T]$ or $[R_T] \ll [G_T]$ approach, respectively.

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