Getting it together: signal transduction in G-protein coupled receptors by association of receptor domains

Dennis J Underwood and Kristine Prendergast

The mechanism of signal transduction by G-protein coupled receptors is unknown. Here, we propose that these receptors signal in a way that is qualitatively similar to that seen in the chemokine and endocrine hormone receptor families; the signal occurs when two domains of the receptor are brought together, although this is not the only requirement for signaling.

Address: Merck Research Laboratories, West Point, PA 19486, USA.

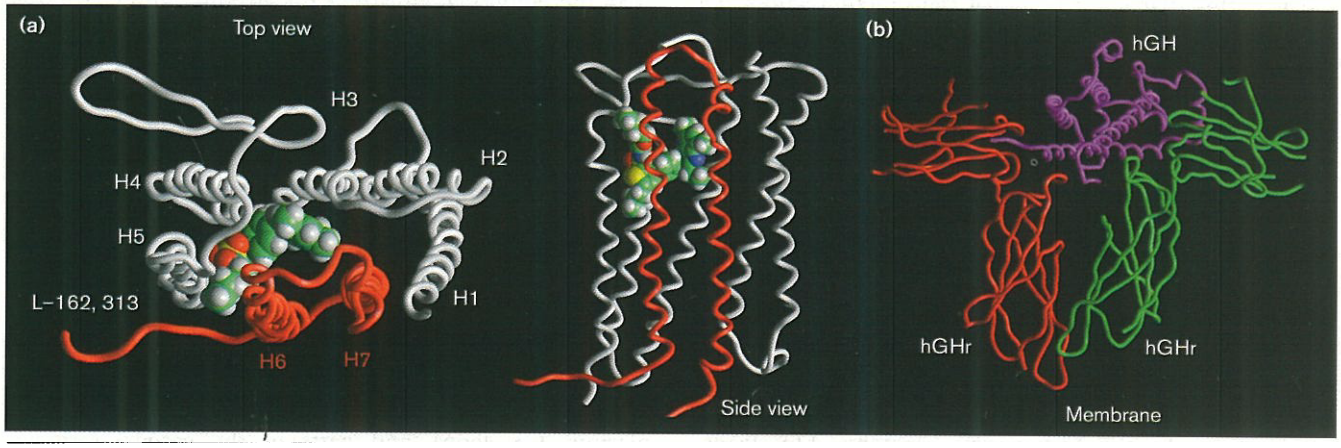
E-mail: dju@merck.com

Chemistry & Biology April 1997, 4:239–248
http://biomednet.com/elecref/1074552100400239

Membranes provide a substantial barrier dividing the intracellular machinery from the surrounding extracellular milieu. Communication between cells and their environment therefore requires transmission of extracellular signals across this barrier [1,2]. Membranes also provide a specialized medium for specific biological processes that are critical for cellular function and the composition of the membrane is important in determining the unique behavior and function of integral membrane proteins. The kinetics and thermodynamics of processes such as signal transduction across membranes, ion diffusion and active and passive transport are inextricably linked to the nature of the membrane. There are two primary reasons for this. First, because the core of the membrane is non-polar, the non-specific hydrophobic interactions that drive associations in an aqueous environment are less important, whereas electrostatic effects are more important. Second, membranes are effectively two-dimensional fluids [3,4], with many of the characteristics typical of liquid crystals [5]. Reactions involving the association of biological components within this environment are augmented by this reduction in dimension; membrane localization increases the rate at which the reactants encounter one another and restricts the orientation in which they collide. This is a frequently-used mechanism of increasing reaction rates, and often involves linking of lipids to proteins to localize them to (or alter their interaction with) the membrane. Examples include the palmitylation of G-protein coupled receptors (GPCRs) [6], palmitylation and myristylation of G-proteins [7] and farnesylation of CAAX boxes [8,9].

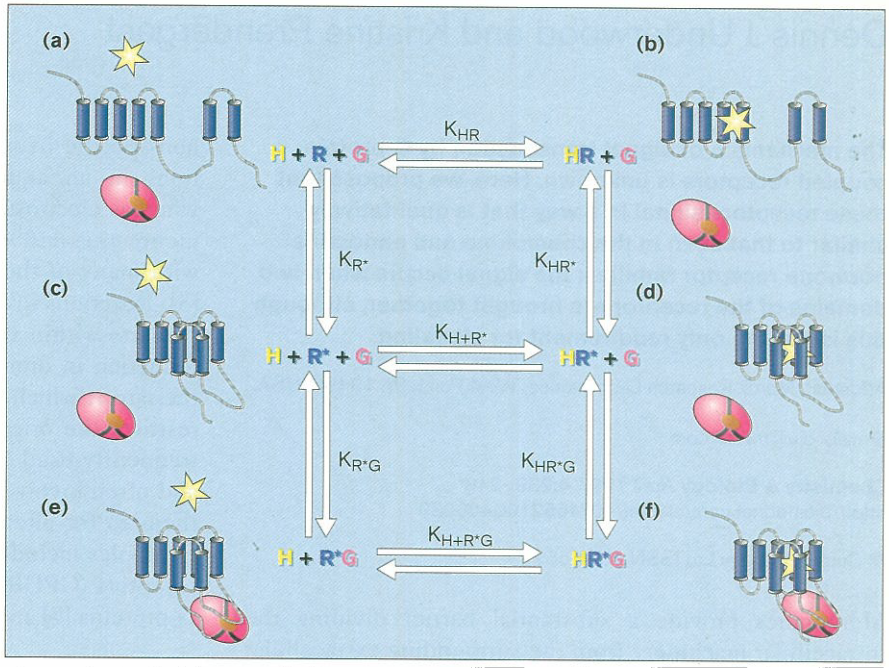
Any model of GPCR signal transduction must address the association of and equilibrium between agonist, antagonist and G-protein, as well as other factors, within the membrane environment. Our proposal is that the transduction of a signal has two elements. We suggest that the association of two domains, transmembrane (TM) helices 1–5 (domain I) and TM helices 6 and 7 (domain II), is necessary for signal transduction (see Fig. 1a). This is not sufficient for signal transduction, however; a second requirement for agonist activity is a specific perturbation of the conformational states of receptor sidechains in the vicinity of the agonist's binding site, followed by G-protein association.

Figure 1



Mechanisms of receptor signaling. **(a)** Top view (extracellular) and side view of a model of the GPCR Aii/AT1, showing a C_α trace of domain I (white) and domain II (red) with the non-peptide agonist L-162,313 (shown as a CPK model) sandwiched between them [40]. Transmembrane helices 1–7 are labeled H1–H7. **(b)** X-ray structure of the soluble domain of human growth hormone receptor (hGHr) with hGH [13]. Two receptor molecules are shown, one in red, the other in green. Between the domains is hGH (magenta).

Figure 2



Description of the proposed receptor states. The seven TM helices are shown in blue, the agonist as a yellow star and the G-protein in majenta. A route through this six state scheme that might describe the lowest free energy path for the signal transduction of most GPCRs is as follows. The first step **[(a)** to **(b)]** is the binding of the agonist to the unorganized, G-protein-free receptor; this step has the equilibrium constant K_{HR}; the free energy of binding is given by $-RT \ln K_{HR}$. In the second step **[(b)** to **(d)]**, a receptor that has already bound agonist rearranges to give an activated receptor. This step has the equilibrium constant K_{HR^*}, determined by the free energy of association of the domains of the receptor. In the third step **[(d)** to **(f)]**, the G-protein binds to the activated receptor. K_{HR^*G} describes the ΔG_{bind} of the G-protein to the GPCR with bound agonist and with the receptor in the activated configuration. Precoupled GPCRs may follow a different route. The transition **(a)** to **(c)**, with equilibrium constant K_{R^*}, may be significantly more favorable for precoupled receptors compared to non-precoupled receptors. (The ratio K_{HR^*}/K_{R^*} is a measure of the efficacy of the agonist H for a particular receptor.) Such receptors may then be able to bind directly to G-proteins **[(c)** to **(e)]**, and this state may represent a local minimum on the free energy manifold for precoupled receptors. K_{R^*G} then describes the ΔG_{bind} of the G-protein to the activated receptor in the absence of bound agonist, and K_{H+R^*+G} the equilibrium which leads to binding of the agonist to the precoupled receptor. Signal transduction occurs from state **(f)**.

Because of the fluidity of the membrane, GPCRs undergo continual and dynamic reorganization restrained only by receptor topology. We suggest that the receptors can access several metastable states which form minima on a complex manifold of equilibria (see Fig. 2) and visit these states even in the absence of G-proteins, agonists and other effectors. When G-proteins and agonists are present, however, the appropriate metastable state is stabilized, and it is this that leads to signal transduction across the membrane. The notion that signaling processes derive from transitions between metastable states of the receptor comes from studies of the light activation of rhodopsin and bacteriorhodopsin [10]. It is expected that the population of receptor states depends on the local concentration and conformation of essential components such as endogenous ligands, G-proteins and ions.

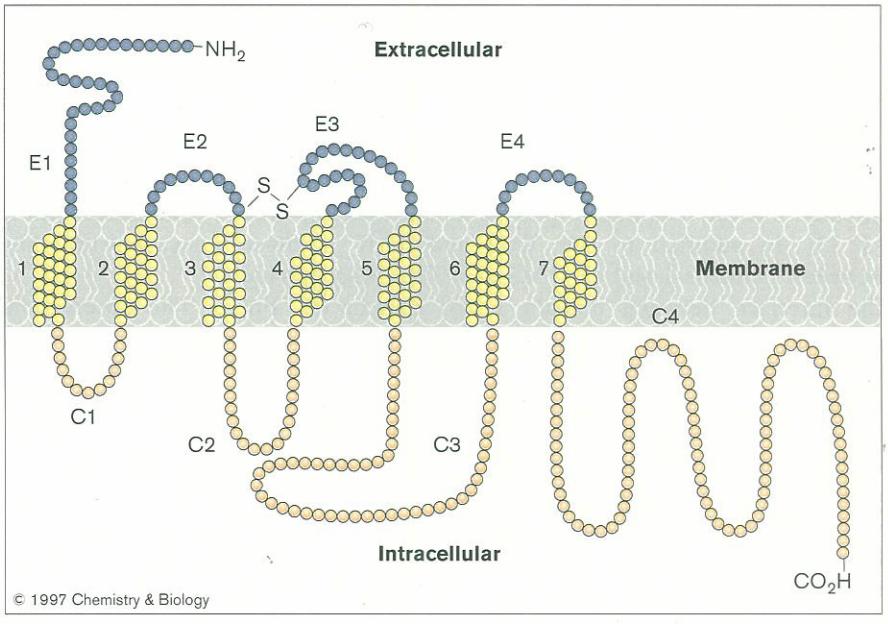
Domain association

Signal transduction processes in the hematopoietic family of integral membrane receptors, which are composed of an extracellular domain, a transmembrane linkage and an intracellular domain, begins by association of the extracellular domains by the endogenous hormone. This leads, through the increased proximity of the transmembrane linkers, to coupling of the intracellular domains [11]. Specific recognition of the coupled intracellular domains by downstream components then initiates the signaling cascade [12]. Experimental evidence for these processes come from structural studies of human growth hormone receptor [13,14]. The cellular response to extracellular growth hormone is initiated by dimerizing the transmembrane receptor (Fig. 1b) to form an intracellular binding site for intracellular kinases of the JAK-STAT pathway [14]. Other receptors that transduce signals by oligomerization include the ciliary neutrotrophic factor receptor [15], the epidermal growth factor receptor family [16], the insulin receptor family, the colony stimulating factor receptor family, the endocrine hormone receptor family and the cytokine receptor family [17–19].

By analogy, we propose that GPCR signal transduction occurs by the association of two tethered domains, domain I and domain II. As well as stabilizing the association of these two domains, agonists perturb specific conformational states of receptor sidechains in the vicinity of their binding sites. This change in conformation is communicated to the cytoplasmic site at which the G-protein binds to the receptor. The G-protein binding site includes the cytoplasmic loop linking TM5 and TM6 (the third cytoplasmic, C3, loop), and parts of the carboxy-terminal domain (the C4 loop), and is formed by the association of TM5, TM6 and TM7 as domains I and II come together. Again, the formation of this site and binding of the G-protein is dependent on the levels of agonist (and

Figure 3

A schematic showing the topology of the receptor domains. The seven helical domains, labeled 1–7, are joined by a series of variable length loops. These are labeled according to their location: E, extracellular and C, cytoplasmic. Domain I is defined as helices 1–5 and domain II as helices 6 and 7.



antagonist), GTP, monovalent and divalent ions and, importantly, the conformational state of the receptor. Agonist and antagonist binding sites are accessible from the extracellular milieu and occupation of these sites affects the distribution of GPCR states.

Topological definition of receptor domains

GPCR topology suggests that the seven helical domains are tethered by a series of variable length loops (see Fig. 3) [20]. These are involved in arranging and constraining the seven helices and, for the cytoplasmic loops, providing part of the binding site for the G-protein trimer. For some GPCRs, the extracellular loops have been identified as contributing to the agonist binding site. The loops joining TM1 to TM2 (C1 loop), TM2 to TM3 (the second extracellular, E2, loop) and TM3 to TM4 (C2 loop) are generally short to medium length. A conserved disulfide bond linking the top of TM3 to the third extracellular (E3) loop at the amino-terminal end of TM5 appears to be important for receptor folding and function, however [6]. We define TM1–5 as domain I in our model.

The loop that joins domain I to domain II, C3, is of variable length, and is known to be critical for receptor function. The C3 and C4 loops are both known to be involved in G-protein binding [21–24]. The E4 loop between TM6 and TM7 is also short to medium in length. In our model the extracellular loops E3 (domain I) and E4 (domain II) are in close proximity. Both of these loops contain components that contribute to agonist binding in at least one tachykinin receptor subtype [25].

Changes in topology do not affect function

Studies show that the functional integrity of GPCRs can be maintained even after removing certain structural constraints. For bacteriorhodopsin, which belongs to the superfamily of seven helix integral membrane proteins but which is not coupled to G-proteins, the loop connecting the segment AB (TM1,2) to CDEF (TM3–7) can be deleted without significantly altering receptor function [26]. Kahn and coworkers [27] fragmented bacteriorhodopsin and measured the change in the thermodynamic stability with respect to retinal binding, finding that the loop structure is not necessary to constrain the helices into a functional receptor. More dramatically, Lefkowitz and coworkers [28] generated functional, split β2-adrenergic receptors by separately expressing TM1–5 and TM6,7 in the same cell. The split receptor bound agonists and antagonists normally, and showed 25% of the activity of wild-type in activation of adenylyl cyclase. Thus, the two segments of the receptor can recombine in the presence of agonist to form a functional, complete receptor. Wess and coworkers [29–31] also reconstituted functional receptors from expression systems which produce mixtures of TM1–5 and TM6,7 domains from different muscarinic receptors (M_2, M_3) and from a mixture of the muscarinic and adrenergic receptors (M_2, α_2) with similar success. These results suggest that the helices of GPCRs have a natural order and a tendency to associate and signal normally even when the structural tether restricting diffusion is broken.

Defining a domain interface: through mutagenesis

Site-directed mutagenesis has provided evidence that a highly conserved acid residue on TM2, involved in Na^+

regulation, is close to a conserved hydrophilic patch on TM7: both sites are implicated in signal transduction [32–34]. Mutation of TM2 Asp79→Asn in the α_{2a} receptor interrupts signal transduction with minimal impact on agonist binding [35]. The angiotensin-ii, AT1 subtype receptor (Aii/AT1) mutations Tyr292→Phe and Asn298→Ala in TM7 indicate that these residues are involved in signal transduction; agonist and antagonist binding are not affected but the coupling to phospholipase C is impaired [32,33]. In addition to the TM2 and TM7 interface between domains I and II, TM5 and TM6 also provide the interface between domain I and domain II. For example, Tyr205 (TM5) in addition to Glu78 (TM2) have been shown to be involved in receptor activation in the neurokinin-1 receptor (NK1) [36]. In the gonadotropin releasing hormone (GnRH) receptor the conserved acid described above is found in TM7 (residue Asp318) [37]. If either Asn87 (TM2) or Asp318 (TM7) are mutated in GnRH, the ligand-binding activity of the receptor is destroyed; but swapping these two residues gives a receptor that can bind ligand, but not signal [38]. Further evidence that TM2 and TM7 interact comes from the observation that muscarinic receptor chimeras were functional only when TM2 and TM7 were from the same subtype [39]. All of these mutations are found at the interface between domain I and domain II and their effect on signal transduction imply that contact between these domains is essential.

Location of the ligand and G-protein binding sites

The footprint of a minimal ligand binding site defined by residues from both domain I and domain II is spatially conserved in a number of GPCR families [6,40,41]. Specifically, for the β2 adrenergic receptor Phe290 (TM6) and Ser204 and Ser207 (TM5) interact with agonists and are required for activating the receptor [42–44]. For larger peptide and protein agonists, the binding site often extends to TM1 and TM2 [6,45,46] or to extracellular loop regions [47] but still preserves contacts with the TM domains.

The third cytoplasmic loop (C3) joining TM5 and TM6 is important in both signal transduction and G-protein binding [21,22,24]. If the C3 loop of the α_{1b} receptor is exchanged for that of β2, both receptors become constitutively active [21,22,24]. The Leu194→Gln mutation in the C3 loop for the Ste3 receptor also causes constitutive activity [23,48]. These studies suggest that the C3 loop is a negative regulatory domain: mutant loops fail to inhibit G-protein binding, resulting in a G-protein–receptor complex with a permanent high affinity agonist binding state. (The secretin class of receptors appears to be more complicated: large portions of both the C3 and the C4 loops can be deleted without affecting G-protein binding and signal transduction [49].) The structural role of this loop in G-protein activation is highlighted by studies of the circular dichroism spectra of free peptides derived from the C3 loop of β receptors. These helical peptides have been shown to activate the G-protein Gs [50]. Further evidence for the involvement of the C3 loop in signal transduction comes from work identifying Tyr254 (C3) as critical for GPCR coupling to the phosphatidyl inositol signaling pathway in muscarinic receptors [51]. This residue is conserved among many different classes of GPCR and communicates with the conserved 'aromatic girdle' implicated in agonist binding and signal transduction [40].

The observation that the C3 loop, identified above as comprising the G-protein binding surface, connects the TM5 and TM6 helices, which are both involved in ligand binding (Fig. 1a), is important because it suggests a direct mechanism for signal transduction. Extracellular ligand binding events will result in a change in the way that the helical rods of the receptor associate, and this can be directly communicated to the intracellular G-proteins through the C3 loop. Conversely, G-protein binding to its intracellular site favors agonist binding.

A minimal model for signal transduction

Precoupled receptors (receptors that have a high affinity for the G-protein in the absence of agonist) have been identified by a battery of experimental approaches (Table 1) [47,52–57]. Examples of receptors showing significant precoupling include the C5a receptor, the adenosine A1 receptor (85–90% precoupled [52]), platelet α_2 receptor (30% precoupled [52]), α_{2B} adrenergic receptor (40% precoupled [53]) and D2 dopamine receptor (40–50% precoupled [52]).

The existence of precoupled GPCRs underscores the importance of including G-protein interactions in mechanistic schemes of signal transduction. There has been much discussion about the two-state receptor model in the recent literature [58–62]. Only two states of the receptor are considered in this model; the activated state from which signal transduction occurs (R*) which is in equilibrium with the resting state (R). In this model agonists bind and increase the population of the activated state. Antagonists drive the equilibrium toward the resting state of the receptor. This description provides a rationale for agonist and antagonist binding but ignores the contribution of G-protein binding to the thermodynamics of ligand binding.

Figure 2 shows a hypothetical set of equilibria between receptor, G-protein and hormone. In this model, agonist or antagonist and G-protein can bind independently to the receptor and stabilize various receptor states including the activated state (R*; see Fig. 2d and Fig. 2e, respectively). The underlying preference of G-proteins for different states of the receptor is governed by complex equilibria, but the whole set of equilibria reduces to the two-state model when individual interactions between the hormone

Table 1

Observation of precoupled states.

Experimental methods	Observations	Mechanism of action and state probed
Kinetics [55,57]	Data fits H•R•G kinetic models.	Implies R•G, precoupled states.
Isolation of R•G [47,56]	Co-purification of G-protein with the receptor in the absence of agonists.	Receptors having intrinsic affinity for G-proteins achieve precoupled states depicted in Fig. 2e.
Chemical cross-linking between R and G [53,54]	EGS is a reagent which cross-links lysine residues. Antagonists, GTP analogs and Na^+ protect against cross-linking.	Negative antagonists, inverse agonists and insurmountable antagonists bind to inactivated receptor (Fig. 2a,b) and thereby shift the equilibria away from activated states (Fig. 2c–f). GTP analogs dissociate G-protein heterotrimers (these equilibria are not shown in Fig. 2) thereby reducing the population of states represented by Fig. 2e,f).
GTP, Gpp(NH)p and GTPγS effects [52,53]	GTP and non-hydrolyzable analogs bind to Gα subunits. These reagents decouple the receptor from the G-protein and thus decrease the binding affinity of agonists.	Probes high affinity (Fig. 2e,f) and low affinity agonist states (Fig. 2a,b, the folded receptor, and Fig. 2c,d, the unfolded receptor) of the receptor. GTP analogs dissociate G-protein heterotrimers these equilibria are not shown in Fig. 2) thereby reducing the population of states represented by Fig. 2e,f). (GTP analogs protect receptors from EGS cross-linking.
Na^+ and monovalent cation effects [34,52,53,80]	Na^+ decouple receptors from the signal transduction events. Na^+ ions cause a concentration dependent decrease in agonist affinity.	Monovalent ions reduce the stability of the precoupled states through a mechanism which is G-protein independent. The conserved TM2 acid has been shown to be the likely site of this interaction. Na^+ protect receptors from EGS cross-linking.
Effects of toxins (PTX, CTX) [53]	Decouple the receptor from the signaling pathway. Diminishes agonist high affinity binding.	Toxins cause ADP-ribosylation of a conserved residue in the carboxy-terminal tail of Gα (Cys in PTX and an Arg in CTX). Thus they suppress (but may not eliminate) precoupling between R and G. Their effect therefore is to diminish states precoupled states such as Fig. 2e,f.
Functional effects by antagonists [53,81]	Addition of antagonists can stimulate cAMP production if the receptor couples through Gi.	The basal activity of receptors (absence of agonist) must occur through a coupled R·G complex (through Fig. 2e but not via 2f). Precoupled receptors inhibiting cAMP through Gi will show stimulation of cAMP with antagonists which diminish precoupled states.

Gpp(NH)p, 5′-guanylylimidodiphosphate; GTPγS, guanosine-5′-O-(3-thiotriphosphate); PTX, pertussis toxin; CTX, cholera toxin; EGS, ethylene glycol bis(succinimidyl)succinate; cAMP, cyclic AMP.

and G-protein with the receptor are ignored. Without these interaction the only relevant states are R and R* and K_{R^*} is modulated by agonist and antagonist binding. From the previous discussion of precoupled receptors, it is clear that the interactions between the receptor and G-proteins are critical, however.

Even the complexity of Figure 2 represents a simplification of the true physiological situation: there are many other factors that are not shown in this model. The population of a given state depends on the details of the interaction between the ligand, receptor and G-protein, not to mention environmental factors such as ionic strength, nucleotide concentrations (GDP and GTP) and membrane factors. Further, each receptor will have a different propensity for association with the G-proteins available in the particular tissue in which it is found [35,63]. Considering the complexity of factors, it is no surprise that the pharmacology for GPCRs is so rich.

Thermodynamic analysis of receptor states

The β2 receptor is not precoupled under most conditions, and so it is clear that the 'ground' state of this system does not involve tight association between the receptor and G-protein. In terms of the simple hypothesis of domain association outlined above, signal transduction in the β2 receptor requires binding of an agonist, organization of the helical domains to provide a G-protein binding site (Fig. 2c), binding of the relevant G-protein and allosteric communication between the agonist and the nucleotide binding site. Enthalpy–entropy compensation studies with the β2 receptor indicate that ΔH correlates linearly with TΔS for a number of agonists, partial agonists and antagonists [64–66] (see also Gilli *et al.* [67]). This is expected since entropy–enthalpy compensation is a general phenomenon for ligand–receptor and enzyme binding. But the binding of agonists, partial agonists and antagonists to the β2 receptor occurs with different compensatory effects (Fig. 4a). Under certain conditions, agonists bind to

GPCRs with a large negative enthalpy change (ΔH_{bind}) resulting in a favorable free energy of binding (ΔG_{bind}); the entropy contribution ($T\Delta S_{bind}$) is unfavorable ($\Delta G_{bind} = \Delta H - T\Delta S$). The balance between favorable enthalpy and unfavorable entropy results from tight evolution of optimal interactions between the receptor and the endogenous agonist. The unfavorable entropy observed for agonist binding to β2 is characteristic of an increase in the overall organization of the system. Antagonists, on the other hand, interact less tightly with less favorable ΔH_{bind} and pay less of an entropic penalty. Partial agonists fall between agonists and antagonists in entropy cost (Fig. 4a) [65,66].

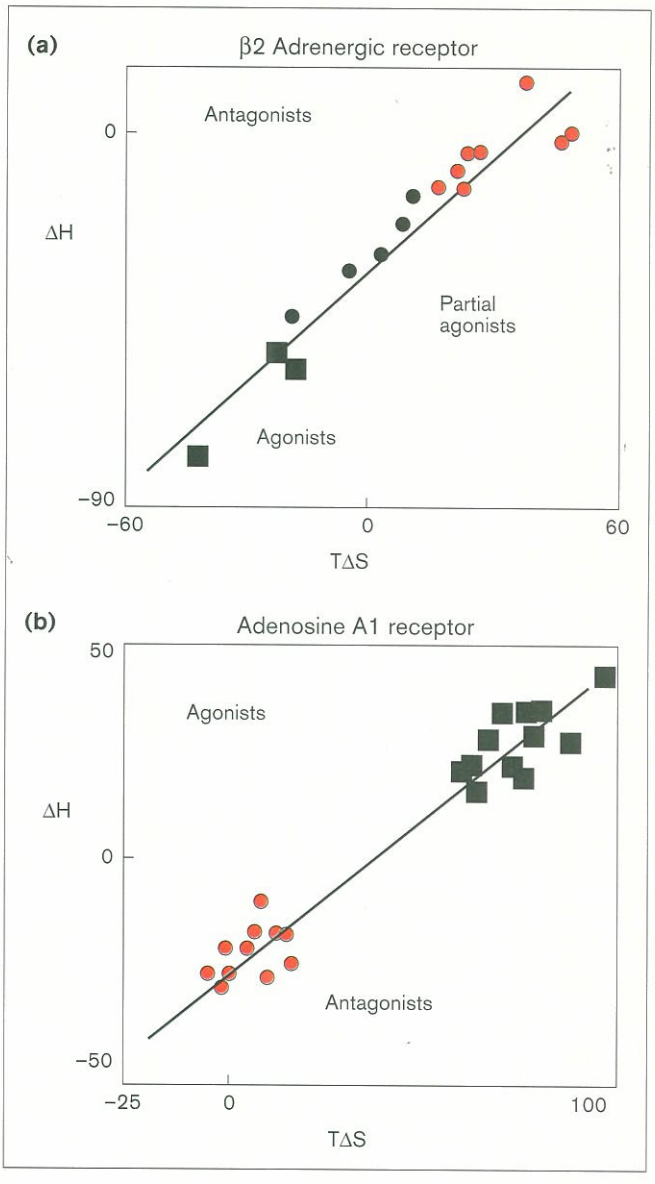
The thermodynamic information for β2 adrenergic receptors in membranes indicate that antagonist binding is temperature independent and entropy driven; this means that ΔG is approximately equal to ΔH, and TΔS is constant [68]. Agonist binding, on the other hand, is temperature dependent and enthalpy driven, so $\Delta G = \Delta H - T\Delta S$. For the high affinity state of the receptor, or in the absence of GTP, there is a large decrease in TΔS. A large decrease in entropy is expected for a process which requires an increase in organization of the system such as the association of agonist, receptor and G-protein (steps described by K_{R^*G} and K_{H+R^*G} shown in Fig. 2). For the low affinity state of the receptor, agonist binding has only a slight temperature dependence. This result is similar for agonist binding in the presence of GTP (the G-protein-decoupled states described by equilibria K_{HR} and K_{H+R^*}; Fig. 2). The smaller decreases in TΔS for agonist binding to the low affinity state or the GTP decoupled state reflect conformational effects that communicate agonist binding to the G-protein. Similar experiments were performed with solubilized receptor in the absence of G-protein [69].

In contrast to the results above, some recent thermodynamic data on the low and high affinity binding states of the β2 receptor indicate that binding of both agonists and antagonists is driven by entropy [70]. But these experiments were performed using tissue-derived cells, not isolated membranes or soluble receptors, and so differences in membrane composition, cellular components and assay conditions may have influenced these results.

Thermodynamic analysis of precoupled receptors

For receptors which have a natural predisposition to fold or preorganize and which are precoupled to G-protein, the binding of agonist does not incur unfavorable entropy changes; for example, the adenosine A1 receptor. Note however, there are still entropic costs of ligand binding and complex changes in solvent organization which will have entropic effects. Nonetheless, the entropic contributions for the precoupled versus the non-precoupled receptors are expected to be different as explained. An example of a precoupled receptor is the adenosine A1 receptor [52]. In its native state, the adenosine A1 receptor has a quite different thermodynamic profile from the β2 adrenergic receptor, showing entropy-driven agonist binding and enthalpy-driven antagonist binding (compare Fig. 4a with Fig. 4b) [67]. But when the receptor is decoupled from the G-protein, its enthalpy–entropy profile becomes similar to that of β2 [71]. Thus, an analysis of receptor interactions with G-proteins is essential to

Figure 4



The thermodynamics of ligand binding are altered in precoupled receptors. **(a)** Graph of the enthalpy versus entropy for a number of agonists, partial agonists and antagonists of the β2 adrenergic receptor, a receptor that is not precoupled to a G-protein. **(b)** Graph of the enthalpy versus entropy for a number of agonists, partial agonists and antagonists of the adenosine A1 receptor, which is a precoupled receptor.

understanding the thermodynamic data for precoupled receptors (Fig. 2).

Ligand-mediated signal transduction requires a specific association of receptor, agonist and the G-protein. Basal level activity occurs in the absence of ligand and is dependent on factors such as receptor reserve (total number of available receptors). The basal activity of precoupled receptors is higher than for non-precoupled receptors [52]; for receptors that are not precoupled the affinity of the receptor for the G-protein in the absence of agonist is low. Precoupled receptors also appear to be less discriminating toward ligands. These receptors accept more ligands as agonists, and the more tightly G-protein-associated receptors are also the more promiscuous. These results suggest that the necessary (but not sufficient) condition of association of domains I and II by the G-protein imparts less stringency on agonist recognition by the receptor.

Both basal activity and constitutive activity [24] are agonist independent: agonists are not required to mediate activation and encounters between the activated receptor R* and the G-protein (Fig. 2e result in GDP/GTP exchange and signal transduction. One method for generating constitutively active receptors is to disrupt the inhibitory function of the C3 loop, thereby allowing the G-protein to bind and promote formation of the activated state of the receptor (R*, Fig. 2c,e) giving an activated receptor complex primed for signal transduction. The precoupled state shown in Figure 2e provides a common structure for understanding phenomena such as basal activity, precoupled receptor promiscuity and constitutively active receptors. These phenomena can be considered as resulting from small structural and conformational changes brought about by mutations and deletions and by changes in environmental conditions that perturb the equilibria between the different receptor states. The manner in which these changes alter the landscape of free-energy peaks and valleys which describe this system of receptor, ligand and G-protein is subtle, complex and difficult to predict; nonetheless, the stabilization of different receptor states has a direct and profound effect on receptor pharmacology.

The six-state model helps explains receptor pharmacology

The two-state model of receptor activation does not explain the above phenomena in a satisfactory way. The simple model that the receptor can be either in the resting or unactivated state (R) or the activated state (R*) offers no explanation for the distinct behavior of precoupled receptor states. Similarly, a four-state model which considers only the interaction with hormones (Fig. 2a–d) cannot account for precoupled states involving G-proteins. The simplest model that includes G-protein interactions is the six-state model depicted in Figure 2. We believe that Figure 2 provides a useful model of GPCR activation processes and helps clarify much of the pharmacology of agonists and antagonists.

The complexity of these interactions is reflected in the richness of ligand pharmacology. Ligands can display agonism, partial agonism, balanced, insurmountable and negative antagonism, or inverse agonism. It has been argued that the phenomenon of inverse agonists supports the two state model of receptor activation [72,73]. In our model inverse agonists sequester the receptor in states that are unable to bind and/or activate G-proteins; these states are represented by Figure 2b. This results in diminished basal activity of the receptor, the hallmark of inverse agonism. The difference between insurmountable antagonists, negative antagonists and inverse agonists may lie more in the degree to which they affect distribution of conformational states within the thermodynamic manifold of the receptor than in grossly different mechanisms of action. For example, ligands that exhibit insurmountable antagonism may have slow off rates [52,53], again sequestering the receptor in states that cannot be rescued by addition of agonist [74]. Antagonists can protect against receptor–G-protein cross-linking reagents such as ethylene glycol bis(succinimidyl) succinate (EGS; see Table 1) [53,54]. For antagonists, a small k_{off} perturbs equilibria and stabilizes states which are non-productive toward signal transduction. Thermodynamics are governed by the law of mass action: the k_{off} for dissociation of an antagonist from the receptor is much smaller than the k_{off} for the agonist [75]. Receptors locked in these states (similar to the states shown in Fig. 2d,e) may present pharmacology typical of insurmountable antagonists and inverse agonists.

Consequences for ligand design

Precoupled receptors have a greater propensity for signal transduction and are readily activated by a greater range of ligands. The key outcome of agonist binding to its receptor is the stabilization of a receptor state that provides a binding site for the G-protein and activates it. Agonist and G-protein binding are cooperative. There are two primary prerequisites for agonism: ligands must have the necessary functionality with the appropriate chirality, and this functionality must be presented to the receptor in a manner specific for receptor activation. Antagonists can prevent a functional response of the receptor in a number of ways: by blocking agonist binding either by partial or full overlap with the agonist binding site (a direct spatial effect) or blocking the binding site; by blocking agonist binding through a conformational or allosteric effect on the receptor (an indirect effect); by interfering directly with the mechanism of signal transduction rather than blocking agonist binding; and by stabilizing conformational states of the receptor which are sequestered from interaction with agonists. Each of these mechanisms provide opportunities to block the functional response of the receptor. A successful strategy is to design

antagonists which bind at the same site as agonists and block the receptor by being unable to present the required arrangement of functionality required for signal transduction. For example, the required functionality can be modified to prevent activation but not binding, or the required geometry for interaction can be destroyed by inverting chirality or blocking appropriate conformations. In either case, balancing potency and selectivity while eliminating agonism can be a challenge. The issue of antagonist development for highly precoupled receptors is more problematic and the strategies are less well developed; the window for achieving antagonism is often small and requires drastic measures such as changing the important stereochemistry of an endogenous agonist or changing the fold of the ligand [76]. Indeed, the structural diversity typical for antagonists may not be apparent for precoupled receptors. Eliminating agonism within a non-peptide drug discovery effort has not always been easy to achieve [40,77–79].

It is not clear how similar our proposal for signal transduction in GPCRs is to that for endochrine hormone and cytokine receptors. It would be satisfying and reassuring to know that there is a common mechanism for signal transduction through the cellular membrane for these very different receptor types and that these similarities were reflected in the thermodynamics. This similarity suggests that the range of receptor pharmacology seen with GPCRs might also be available for the endochrine hormone and cytokine receptors: agonism, partial agonism, balanced, insurmountable and negative agonism, and, perhaps, inverse agonism. The key elements of our hypothesis involve receptor folding and the conformational perturbation of receptor states on agonist binding as necessary and sufficient events for signal transduction. These processes suggest a number of experimental approaches to defining the mechanism of signal transduction in GPCRs: domain linking which couples the receptors in their activated state has been used to demonstrate the need for domain association. The perturbation of conformational states occurring with agonist binding could be studied using fluorescent or paramagnetic probes which are sensitive to the ordering of the receptor. It is also possible that the more stable of the receptor states can be detected and differentiated using biological probes such as antibiotics. If our model is correct, it will have significant ramifications for the design and discovery of ligands as therapeutic agents, especially for receptors that are precoupled to G-proteins. We predict, for example, that it will be relatively simple to find agonists for such receptors but much more difficult to find antagonists. The state of a given receptor will also affect its propensity for signal transduction of receptors and its sensitivity to changes in assay conditions; such considerations will clearly be important in the development of meaningful assays.

References

1. Hollenberg, M. (1990). Receptor triggering and receptor regulation: structure-activity relationships from the receptors point of view. *J. Med. Chem.* **33**, 1275–1281.
2. Lauffenburger, D. & Linderman, J. (1993). *Receptors: Models for Binding, Trafficking and Signaling.* Oxford University Press, New York, USA.
3. Stouch, T. (1993). Lipid membrane structure and dynamics studied by all-atom molecular dynamics simulations of hydrated phospholipid bilayers. *Mol. Simul.* **10**, 317–345.
4. Bassolino, D. Alper, H.E. & Stouch, T.R. (1995). Mechanism of solute diffusion though lipid bilayer membranes by molecular dynamics simulation. *J. Am. Chem. Soc.* **117**, 4118–4129.
5. Collings, P. (1990). *Liquid Crystals.* Princeton University Press, Princeton, USA.
6. Strader, C., Fong, T., Tota, M., Underwood, D. & Dixon, R. (1994). Structure and function of G protein coupled receptors. *Annu. Rev. Biochem.* **63**, 101–132.
7. Hamm, H. & Gilchrist, A. (1996). Heterotrimeric G proteins. *Curr. Opin. Cell Biol.* **8**, 189–196.
8. Reiss, Y., Stradley, S., Gierasch, L., Brown, M. & Goldstein, J. (1991). Sequence requirement for peptide recognition by rat brain p21ras protein farnesyl transferase. *Proc. Natl Acad. Sci. USA* **88**, 732–736.
9. Moores., S. (1991). Sequence dependence of protein isoprenylation. *J. Biol. Chem.* **266**, 14603–14610.
10. Petracchi, D., Lucia, S. & Cercignani, G. (1994). Photobehavior of Halobacterium-Halobium; proposed models for signal transduction and motor switching. *J. Photochem. Photobiol. B: Biol.* **24**, 75–99.
11. Heldin, C.-H. (1995). Dimerization of cell surface receptors in signal transduction. *Cell* **80**, 213–223.
12. Malinin, N., Boldin, M., Kovalenko, A. & Wallach, D. (1997). MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature* **385**, 540–544.
13. De Vos, A., Ultsch, M. & Kossiakoff, A. (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**, 306–312.
14. Wells, J. & De Vos, A. (1996). Hematopoietic receptor complexes. *Annu. Rev. Biochem.* **65**, 609–634.
15. Stahl, N. & Yancopoulos, G. (1994). The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. *J. Neurobiol.* **25**, 1454–1466.
16. Sorokin, A., Lemmon, M. & Schlessinger, J. (1994). Stabilization of an active dimeric form of the epidermal growth-factor receptor by introduction of an inter-receptor disulfide bond. *Biochemistry* **269**, 9752–9759.
17. Banner, D., *et al.*, & Lesslauer, W. (1993). Crystal structure of the soluble human 55KD TNF receptor-human TNF-beta complex: implications for TNF receptor activation. *Cell* **73**, 431–445.
18. Eck, M. & Sprang, S. (1989). Structure of tumor necrosis factor. *J. Biol. Chem.* **264**, 17595–17605.
19. Jones, E., Stuart, D., & Walker, N. (1989). The structure of tumor necrosis factor at 2.6 Å resolution. *Nature* **338**, 225–228.
20. Humblet, C. & Mirzadegan, T. (1992). In *Annual Reports in Medicinal Chemistry* (Bristol, J., ed.), Academic Press Inc., San Diego, USA.
21. Ren, Q., Kurose, H., Lefkowitz, R.J. & Cotecchia, S. (1993). Constitutively active mutants of the alpha-2-adrenergic receptor. *J. Biol. Chem.* **268**, 16483–16487.
22. Samama, P., Cotecchia, S., Costa, T. & Lefkowitz, R.J. (1993). A mutation-induced activated state of the beta(2)-adrenergic receptor - extending the ternary complex model. *J. Biol. Chem.* **268**, 4625–4636.
23. Boone, C., Davis, N.G. & Sprague G.F. (1993). Mutations that alter the 3rd cytoplasmic loop of the a-factor receptor lead to a constitutive and hypersensitive phenotype. *Proc. Natl Acad. Sci. USA* **90**, 9921–9925.
24. Lefkowitz, R.J., Cotecchia, S., Samama, P. & Costa, T. (1993). Constitutive activity of receptors coupled to guanine-nucleotide regulatory proteins. *Trends Pharmacol. Sci.* **14**, 303–307.
25. Fong, T., Huang, R. & Strader, C. (1992). Localization of agonist and antagonist binding domains of the human neurokinin-1 receptor. *J. Biol. Chem.* **267**, 25664–25667.
26. Kahn, T. & Engelman, D.M. (1992). Bacteriorhodopsin can be refolded from two independently stable transmembrane helices and the complementary five-helix fragment. *Biochemistry* **31**, 6114–6151.
27. Kahn, T., Sturtevant, J. & Engelman, D. (1992). Thermodynamic measurements of the contributions of helix-connecting loops and of retinal to the stability of Bacteriorhodopsin. *Biochemistry* **31**, 8829–8839.

28. Kobilka, B., Kobilka, T., Daniel, K., Regan, J., Caron, M. & Lefkowitz, R. (1988). Chimeric alpha2 and beta2 adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* **240**, 1310–1360.
29. Maggio, R., Vogel, Z. & Wess, J. (1993). Coexpression studies with mutant muscarinic adrenergic-receptors provide evidence for intermolecular cross-talk between G-protein-linked receptors. *Proc. Natl Acad. Sci. USA* **90**, 3103–3107.
30. Maggio, R., Vogel, Z. & Wess, J. (1993). Reconstitution of functional muscarinic receptors by co-expression of amino and carboxyl terminal receptor fragments. *FEBS Lett.* **319**, 195–200.
31. Wess, J. (1993). Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol. Sci.* **14**, 308–313.
32. Marie, J., *et al.*, & Bonnafous, J.C. (1994). Tyr(292) in the 7th transmembrane domain of the AT(1a) Angiotensin-ii receptor is essential for its coupling to Phospholipase-C. *J. Biol. Chem.* **269**, 20815–20818.
33. Hunyady, L., Bor, M., Baukal, A., Balla, T. & Catt, K. (1995). A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type-I Angiotensin-II receptor. *J. Biol. Chem.* **270**, 16602–16609.
34. Horstman, D., Brandon, S., Wilson, A., Guyer, C., Cragoe, E. & Limbird, L.E. (1990). An aspartate conserved among G-protein receptors confers allosteric regulation of a2-adrenergic receptors by sodium. *J. Biol. Chem.* **265**, 21590–21595.
35. Chabre, O., Conklin, B.R., Brandon, S., Bourne, H.R. & Limbird, L.E. (1994). Coupling of the Alpha(2a)-Adrenergic receptor to multiple G-Proteins - a simple approach for estimating receptor-G-Protein coupling efficiency in a transient expression system. *J. Biol. Chem.* **269**, 5730–5734.
36. Fong, T., *et al.*, & Strader, C.D. (1994) Mutational analysis of neurokinin receptor function. *Peptide Symp.*, Montreal, Canada.
37. Zhou, W., *et al.*, & Sealfon, S.C. (1994). A reciprocal mutation supports helix-2 and helix-7 proximity in the gonadatropin-releasing - hormone receptor. *J. Mol. Endocrinol.* **45**, 165–170.
38. Cook, J., Faccenda, E., Anderson, L., Couper, G., Eidne, K. & Taylor, P. (1994). Effects of Asn 87 and Asp 318 mutations on ligand binding and signal transduction in the rat GnRH receptor. *J. Mol. Endocrinol.* R1–R4.
39. Pittel, Z. & Wess, J. (1994). Intramolecular interactions in muscarinic acetylcholine-receptors studied with chimeric m2/m5 receptors. *Mol. Pharmacol.* **45**, 61–64.
40. Underwood, D., Strader, C., Rivero, R., Patchett, A., Greenlee, W. & Prendergast, K. (1994). Structural model of antagonist and agonist binding to the Angiotensin-II, AT1 subtype G-protein coupled receptor. *Chem. Biol.* **1**, 211–221.
41. Strader, C.D. (1993). Mapping the ligand-binding sites of G-protein coupled receptors. *Abstr. Papers Am. Chem. Soc.* **206**, 262.
42. Strader, C., Candelore, M., Hill, W., Sigal, I. & Dixon, R. (1989). Identification of two serine residues involved in agonist activation of the b adrenergic receptor. *J. Biol. Chem.* **264**, 13572–13578.
43. Dixon, R., *et al.*, & Strader, C.D. (1989). Genetic analysis of the molecular basis for β adrenergic receptor subtype specificity. *Proteins* **6**, 267–274.
44. Tota, M. & Strader, C. (1990). Characterization of the binding domain of the beta-adrenergic receptor with the fluorescent antagonist carazolol. Evidence for a buried ligand binding site. *J. Biol. Chem.* **265**, 16891–16897.
45. Fong, T.M., Huang, R., Yu, H. & Strader, C.D. (1993). Ligand-receptor interactions at neurokinin receptors. *J. Neurochem.* **61**, 63.
46. Fong, T.M., Huang, R., Yu, H. & Strader, C.D. (1993). Mapping the ligand-binding site of the nk-1 receptor. *Regul. Pept.* **46**, 43–48.
47. Siciliano, S., *et al.*, & Springer, M. (1994). Two-site binding of C5a by its receptor: an alternative binding paradigm for G-protein-coupled receptors. *Proc. Natl Acad. Sci. USA* **91**, 1214–1218.
48. Rao, V., Cohen, G., & Oprian, D. (1994). Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* **367**, 639–642.
49. Nissenson, R.A., Huang, Z.M., Blind, E. & Shoback, D. (1993). Structure and function of the receptor for parathyroid-hormone and parathyroid hormone-related protein. *Receptor* **3**, 193–202.
50. Shinagawa, K., Ohya, M., Higashijima, T. & Wakamatsu, K. (1994). Circular-dichroism studies of the interaction between synthetic peptides corresponding to intracellular loops of beta-adrenergic receptors and phospholipid-vesicles. *J. Biochem.* **115**, 463–468.
51. Bluml, K., Mutschler, E. & Wess, J. (1994). Identification of an intracellular tyrosine residue critical for muscarinic receptor-mediated stimulation of phosphatidylinositol hydrolysis. *J. Biol. Chem.* **269**, 402–405.
52. Tian, W.-N. (1993). Precoupling of Gi/Go-linked receptors and its allosteric regulation by monovalent cations. *Life Sci.* **52**, 1899–1907.
53. Shi, A.-G. & Deth, R. (1994). Precoupling of alpha-2B adrenergic receptors and G-proteins in transfected PC-12 cell membranes - influence of pertussis toxin and a lysine directed cross-linker. *J. Pharmacol. Exp. Therapeut.* **271**, 1520–1527.
54. Kermode, J., DeLuca, A., Zilberman, A., Valliere, J. & Shreeve, S. (1992). Evidence for the formation of a functional complex between vasoactive intestinal peptide, its receptor and Gs in lung membranes. *J. Biol. Chem.* **267**, 3382–3388.
55. Neubig, R.R., Gantzos, R.D. & Thomsen, W.J. (1988). Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: evidence for a precoupled receptor–guanine nucleotide protein complex. *Biochemistry* **27**, 2374–2384.
56. Li, L.-Y., Zhang, Z.-M., Su, Y.-F., Watkins, W. & Chang, K.-J. (1992). Purification of opioid receptor in the presence of sodium ions. *Life Sci.* **51**, 1177–1185.
57. Posner, R., Fay, S., Domalewski, M. & Sklar, I. (1994). Continuous spectrofluorometric analysis of formyl peptide receptor ternary complex interactions. *Mol. Pharmacol.* **45**, 65–73.
58. Chidiac, P. (1995). Receptor state and ligand efficacy. *Trends Pharmacol. Sci.* **16**, 83–84.
59. Kenakin, T. (1995). Pharmacological proteus? *Trends Pharmacol. Sci.* **16**, 256–258.
60. Kenakin, T. (1995). Efficacy as the molecular property of a ligand. *Trends Pharmacol. Sci.* **16**, 84–85.
61. Leff, P. (1995). The two state model of receptor activation. *Trends Pharmacol. Sci.* **16**, 89–97.
62. Milligan, G., Bond, R. & Lee, M. (1995). Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.* **16**, 10–13.
63. Costa, T., Ogino, Y., Munson, P., Onarau, H. & Rodbard, D. (1992). Drug efficacy at guanine nucleotide binding regulatory protein linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.* **41**, 549–560.
64. Searle, M. & Williams, D. (1992). The cost of conformational order: entropy changes in molecular associations. *J. Am. Chem. Soc.* **114**, 10690–10697.
65. Weiland, G., Minneman, K. & Molinoff, P. (1979). Fundamental differences between the molecular interactions of agonists and antagonists with the β-adrenergic receptor. *Nature* **281**, 114–117.
66. Miclavc, A., Kocjan, D., Mavri, J., Koller, J. & Hadzi, D. (1990). On the fundamental difference in the thermodynamics of agonist and antagonist interactions with β-adrenergic receptors and the mechanism of entropy driven binding. *Biochem. Pharmacol.* **40**, 663–669.
67. Gilli, P., Ferretti, V., Gilli, G. & Borea, P.A. (1994). Enthalpy-entropy compensation in drug-receptor binding. *J. Phys. Chem.* **98**, 1515–1518.
68. Contreras, M., Wolfe, B. & Molinoff, P. (1986). Thermodynamic properties of agonist interactions with the beta adrenergic receptor-coupled adenylate cyclase system: I. High- and low-affinity states of agonist binding to membrane bound beta adrenergic receptors. *J. Pharmacol. Exp. Therapeut.* **237**, 154–164.
69. Contreras, M., Wolfe, B. & Molinoff, P. (1986). Thermodynamic properties of agonist interactions with the beta adrenergic receptor-coupled adenylate cyclase system: II. Agonist binding to soluble beta adrenergic receptors. *J. Pharmacol. Exp. Therapeut.* **237**, 165–172.
70. Fraeyman, N., Bazan, A. & Vanscheeuwijck, P. (1994). Thermodynamic analysis of isoproterenol binding to β-adrenoceptors in rat lung membranes. *Eur. J. Pharmacol.* **267**, 63–69.
71. Borea, P., Varani, K., Guerra, L., Gilli, P. & Gilli, G. (1992). Binding thermodynamics of A1-adenosine receptor ligands. *Mol. Neuropharmacol.* **2**, 273–281.
72. Black, J. & Shankley, N. (1995). Inverse agonists exposed. *Nature* **374**, 214–215.
73. Bond, R., *et al.*, & Lefkowitz, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta2 adrenoceptor. *Nature* **374**, 272–276.
74. Schambye, H., Hjorth, S., Bergsma, D., Sathe, G. & Schwartz, T. (1994). Differentiation between binding sites for angiotensin II and non-peptide antagonists on the AT1 receptors. *Proc. Natl Acad. Sci. USA* **91**, 7046–7050.
75. Ruffolo, R. (1993). Drug receptor theory. *Drug News & Perspective* **6**, 12–23.
76. Ward, P., Ewan, G., Jordan, C., Ireland, S., Hagan, R. & Brown, J. (1990). Potent and highly selective neurokinin antagonists. *J. Med. Chem.* **33**, 1848–1851.

77. Perlman, S., Schambye, H.T., Rivero, R.A., Greenlee, W.J., Hjorth, S.A. & Schwartz, T.W. (1995). Nonpeptide angiotensin agonist - functional and molecular interaction with the at(1) receptor. *J. Biol. Chem.* **270**, 1493–1496.
78. Kivlighn, S., *et al.*, & Siegl, P.K.S. (1995). Discovery of L-162,313: a nonpeptide that mimics the biological actions of angiotensin II. *Am. J. Physiol.* **268**, R820–R823.
79. Dezube, M., *et al.*, & Triantafillou, J.A. (1995). Modification of receptor selectivity and functional-activity in cholecystokinin peptoid ligands. *J. Med. Chem.* **38**, 3384–3390.
80. Johansson, B., Parkinson, F. & Fredholm, B. (1992). Effects of monovalent and divalent ions on the binding of the adenosine analog CGS-21680 to adenosine-A2 receptors in rat striatum. *Biochem. Pharmacol.* **44**, 2365–2370.
81. Ma, H. & Green, R. (1992). Modulation of cardiac cyclic AMP metabolism by adenosine receptor agonists and antagonists. *Mol. Pharmacol.* **42**, 831–837.