Multiple Signaling States of G-Protein-Coupled Receptors

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Abstract——Studies have been amassed in the past several years indicating that an agonist can conform a receptor into an activation state that is dependent upon an intrinsic property of the agonist usually based upon its chemical composition. Theoretically, each different agonist could impart its own unique activation state. Evidence for multiple signaling states for the G-protein-coupled receptors will be reviewed and is derived from many different pharmacological behaviors: efficacy, kinetics, protean agonism, differ-

ential desensitization and internalization, inverse agonism, and fusion chimeras. A recent extension of the ternary complex model is suggested by evidence that the different processes that govern deactivation, such as desensitization and internalization, is also regulated by conformers specific to the agonist. Rhodopsin may serve as a primer for the study of multiple activation states. Therapeutic implications that utilize multiple signaling states hold vast promise in the rationale design of drugs.

I. Introduction

Receptor theories try to explain signaling events that occur by the interaction of a ligand with its specific receptor. Although many different theories have evolved, most have origins in the theories of Clark (1937) and the laws of mass action. Clark recognized that the ability of a drug to produce an intracellular signal depended upon the drug ("fixing") to a receptor and to transduce its action upon the receptor. In recent years, our understanding of how drugs bind and subsequently activate receptors is becoming more complex with many different types of conformations resulting from the binding of various ligands or even the same ligand. Although our scientific sophistication has enabled us to detect these multiple signaling states, the important question is whether these different conformational states translate to physiological reality. This chapter will review these activational paradigms in the G-protein-coupled receptors (GPCRs[1]) and provide a review of the prevailing theory that receptors can adopt multiple signaling states.

A. Basic Receptor/G-Protein-Coupling Principles

The GPCR super family includes several thousand distinct but related proteins. They are found in a wide range of organisms and are involved in the transmission of signals across membranes. Over 80% of all hormones signal using these types of receptors. Although the receptors are conserved in structure, the ligands span a large range of vastly diverse entities from peptides,

small molecules, and light. It is estimated that over 5% of the human genome encode for these receptors and represents one of the biggest family of ancestrally related proteins. They are composed of a single polypeptide containing seven regions of 20 to 28 hydrophobic amino acids that represent transmembrane (TM) domains. The TM segments are α-helices, oriented roughly perpendicular to the membrane as shown in rhodopsin (Palczewski et al., 2000). The amino terminus is located on the extracellular side of the membrane and contains several glycosylation sites (Applebury and Hargrave, 1986). The carboxy terminus is located on the intracellular side and contains sites for phosphorylation, which are used in the regulation of the receptor in desensitization and internalization. Three intracellular and three extracellular loops link the TM domains. Most GPCRs also have a highly conserved disulfide bond between the cysteines in the second and third extracellular loops. This bond is needed for proper folding of the protein and the regulation of the high affinity site in binding (Karnik and Khorana, 1990).

The receptors bind a ligand on the extracellular side and following activation by the drug, causes conformational changes that cause the intracellular loops to bind and activate the heterotrimeric G-protein. Some GPCRs may signal through non-G-protein mediated events (for review, see Bockaert et al., 2003). The activated G-protein then dissociates from the receptor, and the various subunits (α and $\beta\gamma$) amplify a second messenger response by activating or inhibiting various effector molecules such as phospholipases, enzymes, or channels. The exact mechanism of the receptor G-protein coupling is still unclear since there is no direct structural information. Based upon the rhodopsin system, it is believed that upon ligand binding, movements of TM3 and TM6 relative to each other are a major force in the activating process and may impart the proper conformation of the intracellular loops for G-protein activation (Farahbakhsh et al., 1995; Altenbach et al., 1996; Farrens et al., 1996; Han et al., 1996). This paradigm seems conserved in other GPCRs, especially the adrenergic receptors. Fluorescence spectroscopic analysis of β_2-adrenergic receptors (ARs) labeled with fluorescent probes that can detect changes in their chemical environment detect movement of both TM3 and TM6 upon agonist binding,

[1]Abbreviations: GPCR, G-protein-coupled receptor; TM, transmembrane; AR, adrenergic receptor; CAM, constitutively active mutant; ICI 118551, ($\pm$)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; PACAP, pituitary adenylyl cyclase-activating polypeptide; AC, adenylate cyclase; PLC, phospholipase C; PLA$_2$, phospholipase A$_2$; 5-HT, 5-hydroxytryptamine; CHO, Chinese hamster ovary; PTH, parathyroid hormone; PTHrP, PTH-related peptide; [^{35}S]GTPγS, guanosine 5'-*O*-(3-[^{35}S]thiotriphosphate); XTP, xanthosine 5'-triphosphate; ISO, isoproterenol; NK, neurokinin; PKC, protein kinase C; RX 831003, 2-(2-*n*-pentyl-2,3-dihydrobenzofuran-2-yl)-4,5-dihydro-1*H*-imidazole; DAMGO, [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin; WT, wild-type; δ-OR, δ-opioid receptor; ICI 174864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; RP, retinitis pigmentosa; EC, extracellular; CGP 20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate; CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one.

similar to the rhodopsin system (Gether et al., 1997; Jensen et al., 2001). Additional nonspectroscopic evidence for movement of TM3 and TM6 in the β_2-AR comes from cysteine reactivity measurements in constitutively active β_2-AR mutants (Javitch et al., 1997; Rasmussen et al., 1999). However, other receptors may show unique differences. Cysteine cross-linking studies in the M3 muscarinic receptor suggest movement of TM5 and TM6 toward each other upon agonist activation (Ward et al., 2002).

II. Receptor Theories

A. Ternary Complex and Modified/Revised Ternary Complex

To understand the potential for multiple states in receptor activation, a brief review of our current understanding of receptor theory is needed. Initially, it was thought that an agonist ligand was the regulator to select or induce an active conformation of the receptor (DeLean et al., 1980). Modern theories have now shifted to receptor states that can exist even without the effects of an agonist. Our current model of receptor theory was first based upon a key paper in which mutations in the third intracellular loop of the β_2-AR resulted in its constitutive activation (Samama et al., 1993). This mutant demonstrated an increased affinity for agonists in the absence of G-protein but not for antagonists with the extent of the affinity increase correlating with its intrinsic activity. Therefore, full agonists displayed large increases in affinity with weaker agonists showing smaller changes in affinity. In addition, the constitutively active mutant (called CAM) exhibits an increased potency of agonist stimulation of second messengers and an increased intrinsic activity for partial agonists. This phenotype resulted in the theory that this mutant receptor might have an increased tendency to adopt an active conformation, which could be responsible for the observed agonist-binding behavior as well as the spontaneous signaling properties. This mutation lead to the revision of the old ternary complex model (DeLean et al., 1980) which postulated that receptor activation required the agonist-promoted formation of an active, "ternary" complex of agonist, receptor, and G-protein.

The revised and extended model (called two-state) includes an explicit isomerization of the receptor first to an active state (R*) before it can couple to the G-protein (Samama et al., 1993). According to this model, constitutive activation has been explained as an alteration of the normal equilibrium between the inactive state (R) and the active state (R*), shifting a higher proportion of receptor molecules in the active R* state. Inverse agonists, previously referred to as negative antagonists such as ICI 118551 for the β_2-AR, have a higher affinity for the inactive state R. Therefore, inverse agonists can reverse a constitutively active phenotype of higher basal activity by shifting the equilibrium of the constitutively active receptor back to the inactive state. Neutral antagonists bind with equal affinity to both R and R*. Therefore, neutral antagonists are unable to shift equilibrium and have no effect on the basal activity of constitutively active receptors. A number of inverse agonists including ICI 118551 and neutral antagonists have been described and verified for the β_2-AR (Chidiac et al., 1994, 1996; Bond et al., 1995) as well as for many other types of GPCRs.

B. Alternative Models

The idea that a receptor can adopt more than one activated R* state was derived from the concept of agonist-directed trafficking of a receptor stimulus to explain the ability of structurally diverse agonists to activate different G-protein-mediated signaling (Kenakin, 1995). According to this model, each agonist is theoretically able to promote its own specific active receptor state, leading to a limitless number of receptor conformations, Rn*. Some of the evidence presented in this review suggests that each ligand may indeed imprint on the receptor a particular but subtle conformation. The criticism of this thinking is that all of these potential conformations might not be physiologically pertinent. In contrast, Leff et al. (1997) proposed a three-state model where the receptor might exist in three states, an inactive (R) and two active conformations (R*, R**), thereby still accounting for multiple G-protein coupling but limiting the number to theoretical physiologically active conformations. Both of these theories (Kenakin and Leff) basically say the same thing, but until we actually know how many physiologically relevant conformations exist, the point is moot.

Another generalization of the revised ternary complex model is called the cubic ternary complex model (Weiss et al., 1996a,b,c). It incorporates all the features of the revised model but differs in that it also allows G-proteins to bind to inactive receptors. This additional feature results in a complete equilibrium description of the three-way interactions between ligand, receptor, and G-proteins. In the revised ternary complex model, a ligand with high affinity for a receptor conformation coupled to G-protein would result in agonist action. In contrast, the cubic ternary complex model implies the existence of a receptor conformation coupled to G-protein, which is unable to evoke a response, allowing a ligand with high affinity for the receptor conformation coupled to G-protein to behave as a neutral antagonist or inverse agonist. This is a distinctive difference of the cubic model compared with the revised ternary complex model. In experimental support of the cubic ternary complex model, tiotidine was found to be an inverse agonist that binds with high affinity to a form of the H2 histamine receptor coupled to Gs that was incapable of signaling. This was documented by showing that in the same cell, tiotidine also impeded the signaling of the β_2-AR system, that is

also coupled to Gs, supposedly by the histamine receptor recruitment of Gs (Monczor et al., 2003).

III. Agonist-Specific Signaling States

A. Evidence from Multiple G-Protein Coupling or Efficacy

One of the first studies to show convincing proof of agonist-specific states was transfection studies using the type-1 pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor. The agonists (PACAP-27 and -38) stimulated adenylate cyclase (AC) with equal potencies, but only PACAP-38 could invoke the inositol phosphate response through phospholipase C (PLC) (Spengler et al., 1993). In subsequent work, the authors document the existence of a new splice variant of the PACAP receptor that was characterized by a 21-amino acid deletion in the N-terminal extracellular domain. They demonstrated that this domain modulates the receptor selectivity with respect to PACAP-27 and -38 binding and controls the relative potencies of the two agonists in phospholipase C stimulation (Pantaloni et al., 1996).

One of the first examples of agonist-specific states from mutational analysis was a Cys to Phe mutation in TM3 of the α_{1b}-AR, a helix-turn below the critical Asp 125 involved in binding the protonated amine of the agonist. This mutation constitutively activates the receptor, resulting in G-protein coupling in the absence of agonist and selective constitutive activation of a single effector pathway [i.e., PLC and not phospholipase A_2 (PLA$_2$)] (Perez et al., 1996). It was shown previously that these two pathways in COS-1 cells are coupled to two different G-proteins (Perez et al., 1993). It was found that phenethylamine ligands (i.e., epinephrine) from full to partial agonists were able to recognize this "selective active state" as determined by binding and potency changes consistent with constitutive activity. However, a series of structurally distinct imidazoline agonists, such oxymetazoline or cirazoline, did not change in either their binding or signaling characteristics. Since Cys was strictly conserved in the β_2-AR, this same mutation was created in the β_2-AR (Zuscik et al., 1998) and gave analogous phenotypes. The β_2-AR C116F mutant selectively, constitutively activated the Na/H exchanger NHE-1 without constitutively activating the Gαs/adenylate cyclase pathway. Both studies indicate that a single receptor subtype forms multiple conformations, different activation states, and that different binding sites exist for different classes of agonists, which promote or induce these specific interactions. This same mutation has been shown to cause similar phenotypes in a cross-section of GPCRs that couple to different G-proteins, such as the angiotensin receptor (Asn 111) (Noda et al., 1996), the CXCR4 chemokine receptor (Asn 119) (Zhang et al., 2002), the platelet-activating factor

receptor (Asn 100) (Ishii et al., 1997), and the bradykinin receptor (Asn 113) (Marie et al., 1999).

In fact, a large area around this residue in TM3 seems responsible for active state isomerization, and many residues may be involved in this mechanism (Parnot et al., 2000), suggesting this area to be a possible "switch region" that can control key steps in the isomerization process. This has been confirmed by spectral studies showing that agonist binding to the β_2-AR induces a conformational change around Cys 125 in TM3 (Gether et al., 1997). There are also analogous residues in rhodopsin (Gly 121) and bacteriorhodopsin (Leu 93) to Cys 128 in the α_{1B}-AR. In rhodopsin, substitution of Gly 121 causes 11-cis-retinal to become a pharmacological partial agonist (Han et al., 1997) allowing the mutant rhodopsin to activate transducin in the dark. Replacement of Gly 121 with residues of increasing size results in increased transducin activation in the presence of the agonist, all-trans-retinal. Replacement of Leu 93 in bacteriorhodopsin results in a 250-fold increase in the time to complete the photocycle with the continued presence of the 13-cis-retinal intermediate (Delaney et al., 1995). Since bacteriorhodopsin's photocycle is opposite that of rhodopsin (proton transport is initiated by the light-induced isomerization from all trans to 13-cis configuration), the 13-cis-retinal build up represents an increase in an active state intermediate. All of these residues are predicted to face the water-accessible binding pocket, and in rhodopsin, the phenotype can be "rescued" by an appropriate substitution in Phe 261 in TM6.

Another example of multiple signaling states from multiple G-protein couplings is seen in the dopamine receptor. The human dopamine D (2long) [D (2L)] receptor was expressed with four different G-proteins in Sf9 cells using the baculovirus expression system. When coexpressed with various G-protein subunits, the receptor displayed a high-affinity binding site for the agonists, which was sensitive to GTP, demonstrating functional interaction between the receptor and the different G-proteins. Comparison of the effects of different agonists in the different preparations showed that each agonist differentially activated the four G-proteins. These results indicate that the degree of selectivity of G-protein activation by the D (2L) receptor can depend on the agonist-specific conformations of the receptor (Gazi et al., 2003).

The notion that a receptor conformation is important in recognizing a G-protein-activated state is also supported by the observation of the uncoupling of G-proteins. In the α_{2a}-AR, a point mutation in TM2 uncoupled the receptor from activating potassium currents but not calcium currents (Suprenant et al., 1992). Since this mutation as well as many CAMs is located in the transmembrane domains and not in the intracellular loops, which are thought to interact directly with the G-proteins, the receptor conformation must have changed to allow this differential coupling to the G-proteins. In

another study, an arginine residue in the 7TM domain of the prostaglandin E receptor uncoupled the receptor from Gs while still maintaining its Gi coupling ability. This study also suggested that the α-carboxylic acid group of the agonist and its interaction with the arginine in TM7 was responsible for this selectivity (Negishi et al., 1995).

Multiple signaling states can also be seen when looking at efficacy differences. A study using both the 5-hydroxytryptamine2A (5-HT2A) and 5-HT2C receptors stably expressed in Chinese hamster ovary-K1 (CHO-K1) cells found ligands to have differing relative efficacies for the two signaling pathways without any difference in potencies (Berg et al., 1998). A recent study in NIH 3T3 cells stably expressing the 5-HT2A receptor was used to explore the capacity of structurally distinct ligands to elicit differential signaling through PLC or PLA$_2$ pathways. The authors also confirm in this study that the two pathways are independent from each other. They employed structurally diverse ligands from the tryptamine, phenethylamine, and ergoline families of 5-HT2A receptor agonists. The data are consistent with the hypothesis of agonist-directed trafficking because many of the ligands were able to display preferential activation of the PLC or PLA$_2$ signaling pathways (Kurrasch-Orbaugh et al., 2003). A similar result of agonist trafficking was also found in the 5-HT1A receptor (Newman-Tancredi et al., 2002). In the parathyroid hormone (PTH)-related peptide (PTHrP) and the PTH/PTHrP receptors, a peptide analog discriminated between the two constitutively active receptor mutants suggesting that the mutant conferred constitutive receptor activity by inducing distinct conformational changes (Carter et al., 2001). Intrinsic activities of different δ-opioid agonists were determined in a [^{35}S]GTPγS binding assay using cell membranes from CHO cells stably expressing the wild-type or a W284L mutant human δ-opioid receptor. The mutation had opposite effects on the intrinsic activities of agonists belonging to different chemical classes. The effects of the mutation on agonist affinities and potencies were independent from its effects on the intrinsic activity of the agonists. The results indicated that δ-opioid agonists of different chemical classes use specific conformations for G-protein activation (Hosohata et al., 2001).

A noteworthy study found a molecular determinant on a ligand that was responsible for the agonist trafficking. Using the cloned octopamine receptor from *Drosophila*, which can couple to inhibition of AC and intracellular calcium release via separate G-proteins, the two full agonists for this system octopamine and tyramine showed opposite potencies in the stimulation of these pathways in CHO cells. In the inhibition of AC, tyramine is about two orders of magnitude more potent than octopamine. However, octopamine is more potent then tyramine in the calcium response. These two agonists differ by only a single hydroxyl, and this alone appears

to be the distinguishing factor for the preferential coupling (Robb et al., 1994).

B. Evidence from Kinetic/Binding Studies

1. Ligands. Using the native β_2-AR cell line S49cyc- and inducing Gs expression, different β_2-AR agonists were measured for their ability to stimulate AC under GTP-limiting conditions and found to have different rates of ternary complex dissociation (Krumins and Barber, 1997). Using a series of weak to full β_2-AR agonists, Seifert et al. (2001) examined their ability at promoting two different steps of the G-protein cycle: 1) stabilizing the ternary complex, and 2) activating GTPase activity. Using the wild-type and a CAM β_2-AR, there was no correlation between efficacy of ligands in activating GTPase versus their ability to stabilize the ternary complex. These results suggest that the receptor conformation that promotes GDP release and GTP binding is different from the receptor conformation that stabilizes the ternary complex, suggesting the presence of multiple intermediate activation states that controls each sequential step of the activation process (Seifert et al., 2001).

2. GTP Analogs. The effects of different purine nucleotides GTP, ITP, and xanthosine 5′-triphosphate (XTP) were examined on receptor/G-protein coupling using a fusion protein of the β_2-AR and the α subunit of the G protein Gs. GTP was more potent and efficient than ITP and XTP at inhibiting ternary complex formation and supporting AC activation. The effects of several β_2-AR ligands on nucleotide hydrolysis and on AC activity were studied in the presence of GTP, ITP, and XTP. The efficacy of agonists at promoting GTP hydrolysis correlated well with the efficacy of agonists for stimulating AC in the presence of GTP. This was, however, not the case for ITP hydrolysis and AC activity in the presence of ITP. The efficacy of ligands at stimulating AC in the presence of XTP differed considerably from the efficacies of ligands in the presence of GTP and ITP, and there was no evidence for receptor-regulated XTP hydrolysis. The findings support the concept of multiple ligand-specific receptor conformations and demonstrated the usefulness of purine nucleotides as tools to study conformational states of receptors (Seifert et al., 1999).

3. Fluorescent and Biophysical Studies. Some of the best evidence for multiple signaling conformations come from the studies of Kobilka and colleagues using purified preparations of the β_2-AR (Ghanouni et al., 2001). Spectral changes are a direct evidence of receptor shifts in conformation resulting from changes in protein-protein contacts and helical movements, since the chemical environment would change around a fluorescent reporter molecule covalently attached to the receptor. To study the mechanism of how different classes of ligands can modulate receptor function, fluorescence lifetime analysis of a fluorophore covalently attached to Cys 265 located in the third intracellular loop at the cytoplasmic

end of the TM6 was used. When the labeled receptor was bound to a full agonist, the intracellular loop domain existed in two distinct conformations. Moreover, the conformations induced by a full agonist were distinguishable from those induced by partial agonists (Fig. 1). Similar to the full agonist isoproterenol (ISO), they observed two lifetimes representing two different receptor conformations around the fluorophore when the receptor was bound to saturating concentrations of salbutamol and dobutamine. The long lifetime component found when the two partial agonists were bound is identical to that observed in the ISO-bound receptor. However, the short lifetime component for the partial agonist-bound receptor is different from that for the full agonist-bound receptor (Ghanouni et al., 2001). These results suggest that each agonist may have its unique spectra and, thus, conformational state. In support, it had also been shown earlier in the β_2-AR that agonists and antagonists can induce distinct conformational states of the receptor (Gether et al., 1995). Ligand-dependent structural changes as measured by fluorescent anisotropy showed that agonists and antagonists have opposite effects on baseline fluorescence.

The two kinetically distinguishable conformational states upon catecholamine binding first recognized by Ghanouni et al. (2001) has now been further dissected. Using a panel of chemically related catechol derivatives, Kobilka identified the specific chemical groups on the agonist responsible for the rapid and slow conformational changes in the receptor (Swaminath et al., 2004). The conformational changes correlated with biologic responses in biochemical assays, suggesting that these conformers were physiologically relevant. Dopamine, which induces only a rapid conformational change, acti-

vates Gs but not receptor internalization. In contrast, norepinephrine and epinephrine, which induce both rapid and slow conformational changes, could activate both Gs and receptor internalization. These studies demonstrate that the endogenous agonist can induce at least two kinetically and functionally distinct conformational states: a rapid state capable of activating Gs and a slow state required for efficient agonist-induced internalization, suggesting that agonist activation follows a series of multiple conformational states with distinct cellular functions (Swaminath et al., 2004). This mechanism would also be conserved to the rhodopsin system. This study is important because it indicates that one endogenous agonist can activate multiple conformers that are physiologically active. Most of the previous work in this field has centered on synthetic agonists each evoking their own conformer, which was always different from the endogenous agonist.

From other laboratories using fluorescent ligands, similar conclusions are being reached. Rapid kinetics of fluorescent neurokinin A (NKA) binding, in parallel with intracellular calcium and cAMP measurements, was used to determine multiple activation states in the tackykinin NK2 receptor. The naturally truncated version of neurokinin A binds to the receptor with a single rapid phase and activates only calcium responses. In contrast, full-length NKA binding exhibits both a rapid phase that correlates with calcium responses and a slow phase that correlates with cAMP accumulation. In addition, activators and inhibitors of protein kinase C (PKC) or PKA exhibit differential effects on NKA binding and associated responses. PKC facilitates a switch between calcium and cAMP responses, whereas activation of PKA diminishes the cAMP responses. Thus, NK2 receptors can adopt multiple active and desensitized conformations with distinct signaling characteristics (Palanche et al., 2001).

Structural changes induced by the binding of agonists, antagonists, and inverse agonists to the cloned δ-opioid receptor immobilized on a solid-supported lipid bilayer were also investigated using plasmon-waveguide resonance spectroscopy. Agonist binding causes an increase in membrane thickness because of receptor elongation, a mass density increase due to an influx of lipid molecules into the bilayer, and an increase in refractive index anisotropy due to transmembrane helix and fatty acyl chain ordering. In contrast, antagonist binding produces no measurable change in either membrane thickness or mass density and a significantly larger increase in refractive index anisotropy, the latter thought to be due to a greater extent of helix and acyl chain ordering within the membrane interior. An inverse agonist produces membrane thickness, mass density, and refractive index anisotropy increases which are similar to, but considerably smaller than, those generated by agonists (Salamon et al., 2002).

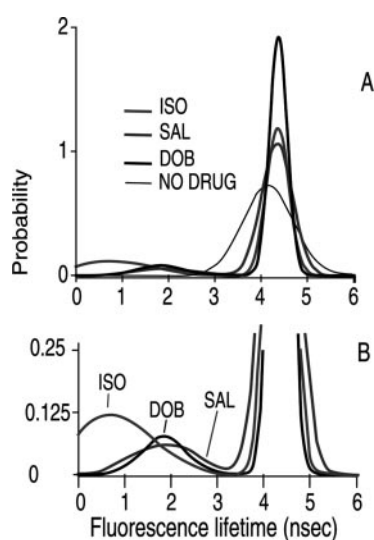FIG. 1. Comparison of the effects of full and partial agonists on the fluorescence lifetime distributions of fluorescein maleimide-β_2AR. A, the effect of the full agonist ISO and partial agonists salbutamol (SAL) and dobutamine (DOB) on the lifetime distributions of fluorescently labeled β_2-AR. B, expanded view of the short-time distributions. Reprinted with permission (Ghanouni et al., 2001).

C. Evidence from Reversal of Efficacy (i.e., Protean Agonism)

1. Native Systems. Interesting and novel ligands exist called "protean" agonists. Proteus was a sea god in Greek mythology and the herdsman of Poseidon's seals who had the ability to change his shape at will. Protean agonists were predicted to exist from theoretical arguments based upon multiple active conformations of GPCRs (Kenakin, 1997). It was predicted that a protean agonist could act both as an agonist or an inverse agonist at the same GPCR. To see this effect, one has to use receptors or tissues that exhibit a high level of constitutive activity. The reversal from agonism to inverse agonism would only occur when an agonist produces an active conformation of lower efficacy than a totally active conformation. Therefore, the higher the constitutive activity, the greater chance to see this other conformation. Gbahou et al. (2003) showed that proxyfan, a high-affinity histamine H3-receptor ligand, acted as a protean agonist at recombinant H3 receptors expressed in the Chinese hamster ovary cells. Using neurochemical and behavioral assays in rodents and cats, proxyfan displayed a spectrum of activity ranging from full agonism to full inverse agonism. Thus, protean agonism demonstrated the existence of alternative agonist active states that was different from the constitutively active state in this system. This was the first report of protean agonism existing for native receptors under physiological conditions (Gbahou et al., 2003).

The coupling of the endogenously expressed α_{2A}-ARs in human erythroleukemia cells (HEL 92.1.7) to calcium mobilization and inhibition of forskolin-stimulated cAMP production was investigated and revealed levomedetomidine to also be a protean agonist. The two enantiomers of medetomidine produced opposite responses. Dexmedetomidine behaved as an agonist in both assays, whereas levomedetomidine, which is a weak agonist in other systems, reduced intracellular calcium levels and further increased forskolin-stimulated cAMP production and was classified as an inverse agonist. Therefore, levomedetomidine was termed a protean agonist because it was capable of activating α_2-adrenoceptors in other systems but inhibited the constitutive activity of α_2-ARs in HEL 92.1.7 cells (Jansson et al., 1998). In a follow-up study in the same cell line, 19 different agonists representing three different structural classes of agonists, catecholamines, imidazoline, and ox-/thiazoloazepine also had differential abilities to activate either the calcium response or the inhibition of AC based their structural class (Kukkonen et al., 2001).

2. Transfected Systems. A double mutant of the rat secretin receptor was studied in which the same mutations produce constitutive activity in the parathyroid hormone receptor. The mutation behaved as predicted, producing mild constitutive activity in the range of 15% of the normal cAMP response in these cells. It bound the natural agonist with almost normal affinity, but rather than promoting agonism, it had become an inverse agonist (Ganguli et al., 1998). For the α_{2A}-AR, two signaling pathways were generated by transfection of two G-proteins, a calcium response mediated by a promiscuous Gα15 protein and a pertussis toxin-resistant [^{35}S]GTPγS binding response mediated by a mutant Gαo Cys351Ile protein. The ligand RX 831003 behaved as a protean agonist, and its activity was highly dependent on the coexpressed Gα protein subunit (Pauwels et al., 2002). Agonist-induced trafficking of the rat neurotensin receptor 1 (NTS1) revealed a reverse potency order between two agonists, EISAI-1 and neuromedin N. The properties of EISAI-1 were also observed in cortical neurons endogenously expressing the NTS1 receptor (Skrzydelski et al., 2003).

The mechanism for the differential regulation of the μ-opioid receptor by agonists was investigated by identifying the receptor domains used to define the relative efficacies of three μ-opioid receptor-selective agonists DAMGO, morphine, and PL017 to inhibit forskolin-stimulated intracellular cAMP production in human embryonic kidney 293 cells. This was accomplished by systematically deleting four to five amino acids clusters within the third intracellular loop of rat μ-opioid receptor, the putative G-protein-coupling motif. Deletion of the four to five amino acid clusters resulted in differential effects on the affinities of the agonists and antagonists and also on the potencies and coupling efficiencies of the three opioid agonists. Thus, these mutational studies suggested that the activation of μ-opioid receptor and interaction between the critical domains within the third intracellular loop and the G-proteins are agonist-selective (Chaipatikul et al., 2003). The study also suggested that differences in the agonist response were due to the relative spatial orientation of the amino acids within the intracellular domain after agonist binding in determining the efficiency of the receptor to activate the G-proteins.

In the β_2-adrenergic receptor, ligands with proven inverse agonism on AC activity were used to see if they could also regulate mitogen-activated protein kinase activation via receptor-mediated scaffold formation. Since scaffolding is not G-protein mediated, the concept of multiple activation states is now diverged outside the realm of G-protein coupling. Despite being inverse agonists in the AC pathway, ICI 118551 and propranolol induced the recruitment of β-arrestin leading to the activation of the extracellular signal-regulated kinase cascade, demonstrating protean behavior. These observations suggest that β-arrestin recruitment is not an exclusive property of agonists and that ligands classified as inverse agonists may rely on β-arrestin for their positive signaling activity. This paradigm was not unique to β_2-AR ligands because the same group also showed that SR121463B, an inverse agonist on the V2 vasopressin receptor-stimulated adenylyl cyclase, also recruited

D. Evidence from Differential Phosphorylation, Desensitization, Internalization, and Palmitoylation

In a very early study before the ternary complex model, there was a lack of cross-desensitization between structurally dissimilar α-adrenoceptor agonists (Ruffolo et al., 1977). Chronic activation of α-ARs with a phenethylamine agonist, such as epinephrine, produced a desensitization from its own activation that could not prevent the activation of the receptor with an imidazoline agonist, such as oxymetazoline and vice versa. This study suggests that there are also multiple agonist-dependent desensitization states. In a more recent example, the equally potent and efficacious agonist ATP and UTP at the P2Y$_2$ purinergic receptor caused differential desensitization with ATP being 10-fold less potent (Velazquez et al., 2000).

The above studies provided evidence in one of the latest developments in the study of multiple signaling states since there also appears to be multiple deactivation states. In retrospect, this was a logical extension of the ternary complex theory, but was not predicted from the model. Since different ligands can invoke different active conformations, it makes sense that the mechanism to deactivate these states may also be different from one another. The divergence in the agonist-receptor conformations have been implicated in the observations that DAMGO but not morphine could induce rapid phosphorylation and internalization of the μ-opioid receptor (Arden et al., 1995; Zhang et al., 1996) and that cAMP-dependent protein kinase could phosphorylate in vitro the μ-opioid receptor in the presence of morphine but not DAMGO (Chakrabarti et al., 1998). Confirming these studies, distinct agonists of the opioid receptors can differentially stimulate receptor phosphorylation and endocytosis (Whistler et al., 1999).

Cholecystokinin receptor antagonists lead to receptor internalization without promoting its phosphorylation (Roettger et al., 1997), and phosphorylation of the angiotensin receptor occurs in a conformation that differs from the active state (Thomas et al., 2000). The data suggested that the AT$_{1A}$ receptor can attain a conformation for phosphorylation without going through the conformation required for inositol phosphate signaling and provide evidence for a transition of the receptor through multiple states, each associated with separate stages of receptor activation and regulation.

Using constitutively active mutants of the human complement factor 5a receptor (C5aR), two different mutant receptors both constitutively activated G-protein-mediated responses, but only one (F251A) was endocytosed in response to agonist stimulation, whereas the other (NQ) was constitutively internalized in the absence of ligand. An inactivating mutation (N296A) complements the NQ mutation, producing a receptor that is activated only upon exposure to agonist, but this double mutant (NQ/N296A) is nevertheless constitutively endocytosed. Thus one mutant (F251A) requires agonist for inducing endocytosis but not for activation of the G-protein signal, whereas another (NQ/N296A) behaved in the opposite fashion (Whistler et al., 2002).

Two mutant forms of the PTHR, H401 and H402, were used which contain substituted histidine residues at positions 401 and 402 in TM6, along with a naturally present histidine residue at position 301 in TM3. Both mutant receptors showed inhibition of PTH-stimulated inositol phosphate and cAMP generation in the presence of increasing concentrations of zinc. However, the mutants showed no zinc-dependent impairment of phosphorylation by G-protein-coupled receptor kinase-2. Likewise, the mutants were indistinguishable from WT PTHR in the ability to translocate β-arrestins to the cell membrane and were also not affected by sensitivity to zinc. These results suggest that agonist-mediated phosphorylation and internalization of PTHR require conformational changes of the receptor distinct from the second messenger active state. Furthermore, PTHR sequestration does not appear to require G-protein activation (Vilardaga et al., 2001).

Palmitoylation of the vasopressin receptor (V1aR) occurs within the Cys 371/Cys 372 motif located in the proximal C-terminal tail domain. Substitution of these residues in a [C371G/C372G] V1aR construct effectively disrupted receptor palmitoylation. The WT V1aR palmitoylation regulated both phosphorylation and sequestration of the receptor and were all regulated by arginine vasopressin. However, the palmitoylation-defective construct [C371G/C372G] V1aR exhibited decreased phosphorylation compared with WT V1aR under both basal and arginine vasopressin-stimulated conditions and was sequestered at a faster rate. In contrast, the binding of four different classes of agonist and intracellular signaling were not affected by palmitoylation. This study suggests that there are different conformational requirements for signaling, agonist-induced phosphorylation, and sequestration of the V1aR (Hawtin et al., 2001).

E. Evidence from Inverse Agonism

A study assessed the effects of short-term treatment (30 min) with inverse agonists on receptor protein levels and on the ability of agonists, inverse agonists, and neutral antagonists to bind to the human δ-opioid receptor (δ-OR). Incubation of human embryonic kidney 293 cells stably expressing δ-OR with the inverse agonist ICI 174864 induced reciprocal changes in agonist and inverse-agonist binding. The binding sites for agonists were reduced by 57%, whereas binding density for the inverse-agonist increased by 44%. In contrast, total receptor protein and sites labeled by neutral antagonists remained unchanged. Spontaneous recovery of maximal agonist binding density after inverse-agonist treatment was slow, suggesting a decrease in the isomerization

rate between the agonist- and inverse agonist-preferring conformations. Overall, the data presented are consistent with the idea that δ-ORs exist in multiple active states capable of discriminating among ligands of different efficacies. They also indicate that after short-term treatment with an inverse agonist, the receptor ability to adopt conformations preferentially induced by ligands is reduced (Pineyro et al., 2001).

F. Evidence from Fusion Chimeras

The first GPCR-G-protein fusion was a coupling of the β_2-AR and Gs (Bertin et al., 1994). The selective coupling of the receptor to the G-protein was first thought to increase the proportion of receptors in the high affinity state, but turned out not to be the case. It is thought that the long C-tail of the receptor allows some coupling to endogenous G-proteins. In one study, fusion proteins between the neurokinin receptor (NK1) and Gq or Gs, respectively, were used in conjunction with truncated C-tails of the receptor in an attempt to exclude interactions with endogenous G-proteins. These tail-truncated fusion proteins gave agonist binding profiles corresponding to two different high affinity states of the receptor (Holst et al., 2001).

Two constructs encoding the human μ-opioid receptor fused at its C terminus to either $G\alpha o_1$ or $G\alpha i_2$ were expressed in *Escherichia coli* and maintained high-affinity binding of the antagonist diprenorphine. Affinities of the μ-selective agonists morphine, DAMGO, and endomorphins as well as their potencies and intrinsic activities in stimulating [35S]GTPγS binding were assessed in the presence of added purified Gβγ subunits. In the presence of Gβγ dimers, the affinities of DAMGO and endomorphin-1 and -2 were higher at the $G\alpha i_2$ fusion protein than $G\alpha o_1$, whereas morphine displayed similar affinities at the two chimeras. Potencies of the four agonists in stimulating [35S]GTPγS binding at the $G\alpha o_1$ chimera were similar, whereas at the $G\alpha i_2$ chimera, endomorphin-1 and morphine were more potent than DAMGO and endomorphin-2 (Stanasila et al., 2000).

IV. Lessons from Rhodopsin

A. Structural Basis for Mechanism of Activation in a G-Protein-Coupled Receptor, Mammalian Rhodopsin

Structural work on the mammalian light receptor rhodopsin began with the experimental determination of the primary structure of the polypeptide (Hargrave et al., 1997) and the elucidation of the protein secondary structural motif consisting of seven antiparallel transmembrane helices. The 7TM structural fold was first identified in bacteriorhodopsin (Henderson and Unwin, 1975). Soon the cDNA-derived polypeptide sequences of β-adrenergic and muscarinic receptor were found to possess a 7TM-fold similar to that adopted by rhodopsin. This lead to a common practice of derivation of primary structures of membrane proteins from their gene sequences, which enabled the identification of a large number of GPCRs where the secondary structures could be modeled on rhodopsin secondary structure. Thus, the seven transmembrane helical motif came to be recognized as the common structural theme in the GPCR superfamily. An informative low resolution structure of rhodopsin was obtained by electron microscopy (Schertler and Hargrave, 1995), and the recent determination of the inactive state (Palczewski et al., 2000) X-ray crystal structure represents an important advance (Fig. 2A). Here, we try to examine a variety of functional studies on rhodopsin activation, which substantiate a crude but consistent picture of the specific movement of TM helices as the basis for activation of function.

B. Lessons from Activation-Induced Events in the Rhodopsin Molecule

The molecular mechanism of ligand activation is best shown in rhodopsin and related visual pigments. They contain the covalently bound light-sensing chromophore 11-*cis*-retinal, which is an inverse agonist. Ligation of opsin with 11-*cis*-retinal sets the molecule to complete inactive state from the native opsin which is in a partially active state. Absorption of a photon causes 11-*cis*-retinal to isomerize to an agonist all-*trans*-retinal. The agonist-ligated opsin displays a series of distinct protein conformational changes, each state with distinct biochemical function, which have been found to be similar to conformational changes identified in bacteriorhodopsin by high-resolution crystallography (Fig. 3) (Sakmar, 2002). These distinct conformational changes are analogous to distinct agonist-specific states found in other GPCRs.

C. Steric Changes in Chromophore

The photolysis pathway of rhodopsin has been known to follow a series of photointermediates which indicates a dynamic change in chromophore-opsin interaction associated with receptor activation and resetting of the activated receptor state to ground state. Detailed optical, resonance Raman, fluorescent infrared, and NMR studies indicate two important structural changes in the chromophore: 1) the isomerization of 11-*cis* to -*trans* bond abolishes an interaction of the bulky 9-methyl group of retinal with the α-carbon atom of the Gly 121 in TM helix 3 and Phe 261 in TM helix 6 (Fig. 2B); 2) the isomerization increases the distance between the two ends of retinal, consequently triggering protein movement (Rao and Oprian, 1996).

D. Electrostatic Changes in Opsin

At least six specific key individual chemical groups in rhodopsin change upon activation. The protonated retinal-opsin Schiff base link forms an ion pair with the counter ion residue Glu 113 in the inactive state. In the activated receptor, the Schiff base is neutral because it is deprotonated and Glu 113 is protonated. Protonation
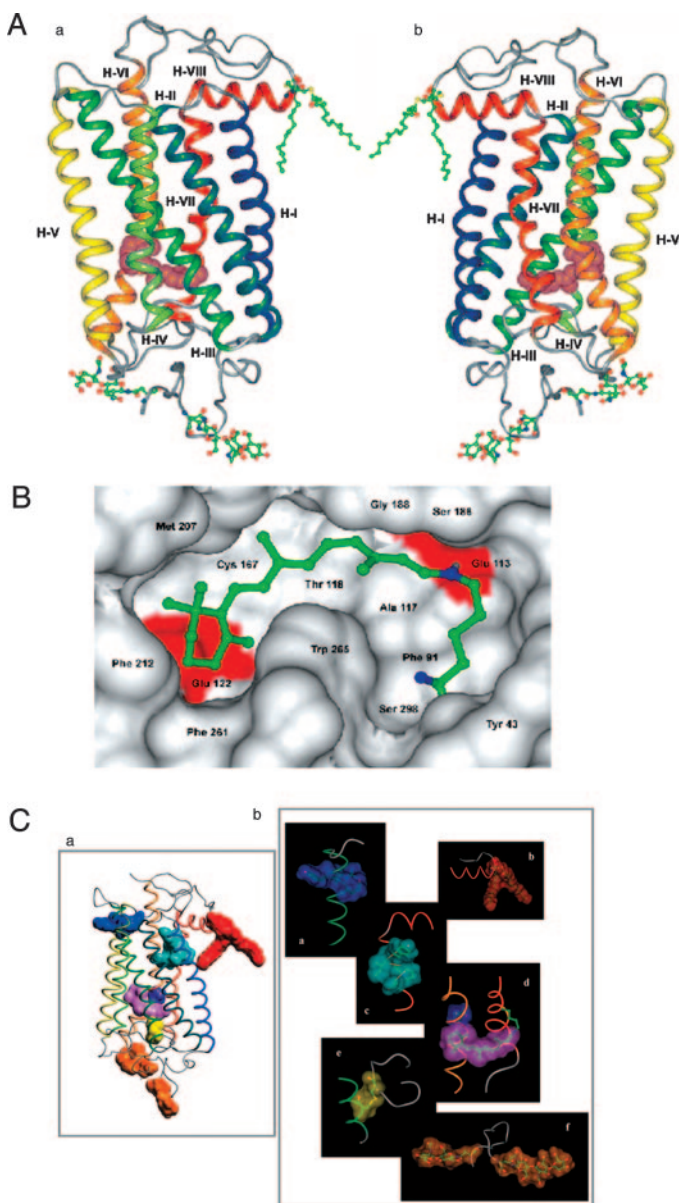
FIG. 2. Rhodopsin structure and function. A, ribbon drawings of rhodopsin. Helices I–VIII are colored as a spectrum of visible light from blue (helix I) to red (helix VIII), and two orientations are shown. Palmitoyl chains and oligosaccharide groups shown using ball-and-stick models. B, vicinity of retinal in the binding site of rhodopsin. The structure of 11-*cis*-retinal bovine opsin using space-filling model. In blue are nitrogen atoms of the peptide bond and the Schiff base, with the hydrogen between Lys 296 and the retinal in green. In red are two acidic residues in the binding site, Glu 113 and Glu 122, which is close to the -ionone ring. C, functional domains of rhodopsin that are highly conserved among members of the GPCR superfamily. a, location of these domains in the three-dimensional structure of rhodopsin. b, close-up of the critical regions: DRY region (a, panel A, in rhodopsin ERY, blue); palmitoyl groups (b, panel A, red); NPXXY region (c, panel A, light blue); chromophore; Pro kink in helix VI, Lys 296 (d, panel A, violet); disulfide bridge (e, panel A, yellow); and oligosaccharide moieties (f, panel A, brown). Reprinted with permission (Filipek et al., 2003).

of four other residues, Asp 83 (TM2), Glu 122 (TM3), His 211 (TM5), and Glu 134 (cytoplasmic end of TM3) change during activation. The steric and electrostatic changes in the retinal-binding pocket of rhodopsin are thought to cause changes in relative disposition of spe-

cific TM helices (Fig. 2B). Signal transmission from the membrane-embedded retinal pocket to the cytoplasmic surface of the receptor requires this repositioning of TM helices (Rao and Oprian, 1996; Sakmar, 2002).

E. Specific Transmembrane Helical Movements in Opsin

Mutagenesis of residues in TM3 and -6, introduction of a metal ion chelation site between TM3 and -6, and disulfide cross-linking TM3 and -6 via substituted cysteine residues provided initial evidence that light-induced movement of these two TM helices is required for G-protein activation (Rao and Oprian, 1996; Sakmar, 2002; Hubbell et al., 2003). Comprehensive cysteine scanning mutagenesis in all the cytoplasmic loops followed by spin labeling and EPR spectroscopy showed specific light-induced movements in the helices in the TM domain were required for a conformational change in the cytoplasmic face. For example, a disulfide bond between Cys 139 (cytoplasmic loop 2) and Cys 248 (Cys 249 or Cys 250 in cytoplasmic loop 3) abolished G-protein activation (Farrens et al., 1996). Thus, relative movements between TM helices 3 and 6 were required for activation. Furthermore, spin-labeling studies using pairs of cysteines showed changes in magnetic interactions in every pair in going from dark to light, indicating changes in distances between the spin labels. These results showed that helices 5 and 6 tilted. Additional movements in other helices were found later on the activation time scale. Separation of TM helices 3 and 6 is now recognized as a general mechanism of GPCR activation.

F. Theory for Activation-Induced Conformations

G-protein activation is achieved by changing an inactive receptor conformation to active conformation, which is mediated by light (agonist) in the rhodopsin receptor. In rhodopsin, the 11-*cis* bond conformation is the "inactive" state and the 11-*trans* is the "active" state. Any group that undergoes a specific chemical change and any amino acid chain that is involved in protein conformational change must obey two different states, which can be designated inactive or active depending on whether the particular state is associated with the active or the inactive receptor. The binary state model can explain a generation of multiple biochemically distinct conformational states because the protein activation involves changes at different topological locations through temporal progression of conformational changes. For instance, let's say that 10 amino acids in a GPCR are involved in the conversion of the inactive to active state conformation. Therefore, each of the 10 amino acids has two distinct states. The number of distinct conformations that may be theoretically possible is 2^{10}. This would account for the large number of residues and/or conformations that are beginning to be discovered in the GPCRs. In rhodopsin, electrostatic and/or steric changes
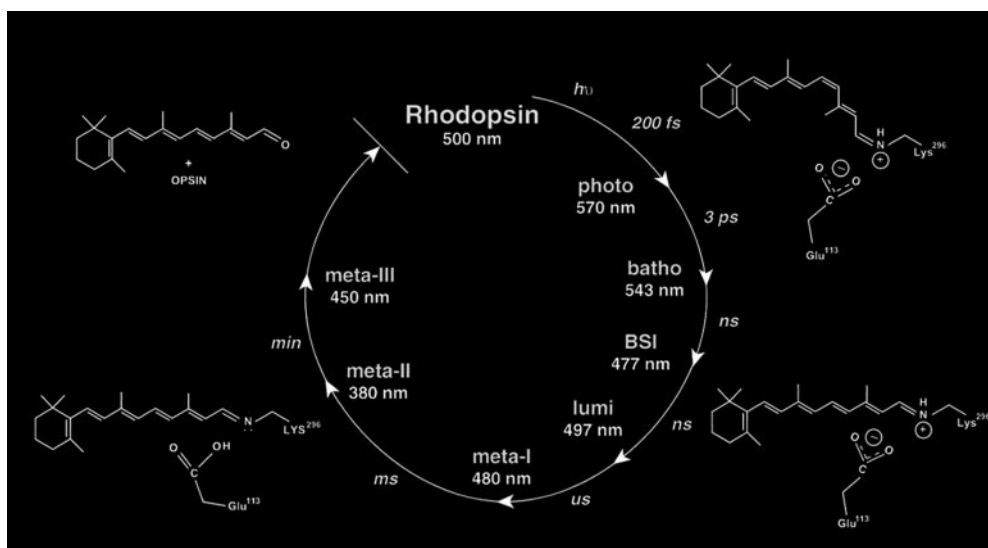
FIG. 3. The Rhodopsin photocycle. Photoisomerization of the chromophore to its 11-*trans* form is the only light-dependent event in vertebrate vision. Photoisomerization in Rho occurs on an ultrafast time scale with photorhodopsin as the photoproduct formed on a femtosecond time scale. The photolyzed pigment then proceeds through a number of well characterized spectral intermediates. As the protein gradually relaxes around 11-*trans*-retinal, protein-chromophore interactions change and distinct max values are observed. Reprinted with permission (Menon et al., 2001).

of individual amino acids to the active state in the chromophore binding pocket generates the early photocycle intermediates in which the G-protein activation domain is not locked into its active state conformation. Time-resolved transition in the G-protein activation domain occurs independently in the late photocycle intermediates. Thus, a combined transition of all groups may create an overall active conformation. This does not mean that all of the observed changes are essential; changes in a minimal group of residues are critical to govern transition to active receptor conformation.

The functional hierarchy of individual groups/residues become obvious in receptors modified due to gene mutations, site-directed mutations, and chemical modifications. The state of individual groups or residues can be altered or even locked into one of the two states. The receptor activation pathway could be specifically altered such that certain transitions become uncoupled from other transitions, often uncovering novel and unsuspected intramolecular events/effects. Thus, the principle of binary transition of groups and residues provides a framework for analyzing the functional alteration in receptors modified, especially those by disease process.

G. Lessons from Gain of Function Rhodopsin Mutations

A complete discussion of activating mutations of rhodopsin is available (Rao and Oprian, 1996). A retinal degeneration mutation in humans alters the Schiff base lysine to glumatic acid (K296E). The corresponding mutant opsin recombinantly expressed in cultured cells causes constitutive G-protein activation by the opsin without binding retinal. The G90D mutation causes congenital night blindness, a defect in scotopic vision in which the dark noise of rod cells is increased in humans.

This mutant expressed in cells indeed displays high constitutive activity, presumably due to intramolecular competition between normal counterion residue Glu 113 and Glu 90 to form an ion pair with the Schiff base (Rao and Oprian, 1996). The basic defect in these examples is the inability of mutants of the light receptor to conform to the inactive state.

H. Lessons from the Mechanism of Loss of Function Caused in Retinitis Pigmentosa Mutations

Retinitis pigmentosa (RP) is a group of hereditary progressive blinding diseases with variable clinical presentations (Rao and Oprian, 1996; Sakmar, 2002). One form of the disease, autosomal dominant RP is linked to mutations in the human rhodopsin gene. Over 100 autosomal dominant RP mutations are known to date. The RP mutations cause partial to total misfolding of the corresponding opsins when their genes were expressed in COS cells. The correctly folded portions of the opsin isolated from COS cells bound 11-*cis*-retinal to form rhodopsin-like chromophore and contained the native Cys 110/Cys 187 disulfide bond. The incorrectly folded opsin was shown by mass spectrometry to contain an abnormal disulfide bond between Cys 185 and Cys 187 (Karnik et al., 1988; Hwa et al., 1997, 2001). In native rhodopsin, two cysteines, Cys 110 and Cys 187, which are disulfide bonded, have been shown to be essential for rhodopsin function, i.e., the stability of the activated metarhodopsin II state formed upon light activation.

Inactivation of rhodopsin by RP mutations in the extracellular (EC) domain is consistent with mutagenesis and structural studies, which demonstrate extensive interaction of the EC loop2 with the retinal. A current model for rhodopsin activation suggests that EC loop2 and its interaction with the chromophore plays a critical

role in the agonist-induced TM helical movements and that this may require the conserved disulphide bond (Yan et al., 2003).

I. Rhodopsin as the Primer

The use of rhodopsin as a primer for agonist-specific states is pertinent for the growing list of GPCRs. Functional domains of rhodopsin are highly conserved among members of the GPCR superfamily. In particular, critical regions of GPCR activation and structure such as the DRY region, palmitoyl groups, NPXXY region, binding pocket, Pro kink in helix VI, the disulfide bridge, and oligosaccharide moieties are highly conserved (Fig. 2C) (Filipek et al., 2003). This suggests a somewhat conserved activation mechanism that may involve common helices and movements, with specifics evolving from a GPCR's particular function. The spectral properties of the chromophore in rhodopsin allow the detection of these activational intermediates. In other GPCRs, sensitive methods for detection of these intermediates are not forthcoming and remain problematic.

V. Therapeutic Implications

G-protein-coupled receptors represent the largest class of drug discovery targets. Although novel agonists and blockers are being developed to allow for receptor discrimination, the concept of multiple activation states will lead to the future development of drugs that are precise for the particular active state that imparts the specified downstream effect. Below are three examples of where the knowledge of the particular activated state may help develop drugs for their therapeutic intervention.

A. G-Protein-Coupled Receptor Diseases Caused by Unregulated Internalization

From the work of Swaminath et al. (2004), different conformations of the activated receptor were responsible for G-protein coupling and receptor internalization. Understanding the internalization-specific conformer may lead to the development of drugs that inhibit this process and lead to functional receptor when a constitutively internalized receptor is responsible for a disease. In the vasopressin receptor, there is a naturally occurring loss of function mutation Arg137His, which is associated with familial nephrogenic diabetes insipidus and induces constitutive arrestin-mediated desensitization and internalization. Arginine 137 is found at the cytoplasmic end of TM3 in a highly conserved GPCR motif (DRY/H). Mutation of a single cluster of three serine residues in the tail of the receptor mutant reversed in the constitutive internalization and promoted high surface expression of the receptor. These findings suggest that unregulated internalization can result in a GPCR-based disease, implying that pharmacological targeting of GPCR internalization without affecting its activa-

tional abilities may be therapeutically beneficial (Barak et al., 2001). Further, therapeutics in this regard could benefit Parkinson's disease (dopamine receptor), heart failure (β_1-ARs), and asthma (β_2-ARs) (January et al., 1998) and optimize the opposite effect of promoting internalization with the case of the CCR5 receptors and AIDS infectivity (Mack et al., 1998).

B. Differential Use of β-Adrenergic Receptor Blockers

Studies with classical β-AR blockers used in clinical medicine has led to the theory that there are two different agonist conformations of the human β_1-AR, resulting from two different binding sites. One site is where classic agonists such as catecholamines (i.e., epinephrine and norepinephrine) and antagonists act and another separate site where some β-blockers have agonist properties and is relatively resistant to competition by other β-AR antagonists. In one study, the cAMP response element and regulated gene transcription was used to confirm the presence of these two β_1-AR conformations and to provide strong evidence that a range of clinically used β-AR blockers may exhibit differential action depending upon their site of interaction at these two sites. It was found that CGP 20712A and atenolol act as classic antagonists at the catecholamine binding site but have much lower affinity for the secondary CGP 12177 site. CGP 12177 and carvedilol are potent antagonists at the catecholamine site but mediate substantial agonist activation of gene transcription via the secondary antagonist-resistant site at higher concentrations. Potential agonist effects of β-blockers were not restricted to this secondary site, and it was shown that some acebutolol and labetolol act primarily via the catecholamine site, whereas others such as pindolol and alprenolol can stimulate both. The different responses to β-blockers seen in the clinic may be caused in part by these agonist responses and the differential activation of the two sites or conformations (Baker et al., 2003). The novel agonist conformation induced by carvedilol, while the classic catecholamine site is desensitized, may suggest a mechanism for why it is a better therapeutic for heart failure (Poole-Wilson et al., 2003).

C. Morphine Dependence and Tolerance

μ-Opioid receptors mediate the principle site of analgesic action induced by morphine. Prolonged use of morphine causes tolerance and dependence. Whereas morphine induces dependence, methadone is used in the treatment of opioid addiction, despite being a full agonist at the μ-receptor. Buprenorphine is also used for therapy and is a partial agonist. To investigate the molecular basis of tolerance and dependence, the cloned mouse μ-opioid receptor was stably expressed, and the effects of prolonged opioid agonist treatment on receptor regulation was examined. Pretreatment of cells with morphine or DAMGO resulted in no apparent receptor desensitization as assessed by opioid inhibition of fors-

kolin-stimulated cAMP levels but resulted in a 3- to 4-fold compensatory increase in forskolin-stimulated cAMP accumulation. Pretreatment with methadone or buprenorphine abolished the ability of opioids to inhibit adenylyl cyclase. No compensatory increase in forskolin-stimulated cAMP accumulation was found with methadone or buprenorphine, and these opioids blocked the compensatory effects observed with morphine and DAMGO. Taken together, these results indicate that methadone and buprenorphine interact differently with the μ-receptor than either morphine or DAMGO and induces different conformational states that affect the desensitization of the receptor. The ability of methadone and buprenorphine to desensitize the μ-receptor and block the compensatory rise in forskolin-stimulated cAMP accumulation may be an underlying mechanism by which these agents are effective in the treatment of morphine addiction (Blake et al., 1997).

D. Use of Stimulation-Biased Assay Systems

Stimulation-biased assay systems have been suggested as a means to detect and screen for drugs specific for a particular active state and signaling pathway (Watson et al., 2000) or for the identification of ligands for orphan receptors. This is achieved by coexpression of a particular G-protein and the use of constitutively active receptors to selectively enrich the system for the desired pathway. Ligands would then be screened for their ability to increase or decrease the signal. In experimental studies, transient transfection of the cyclic AMP response element with a luciferase reporter together with the cDNA for the parathyroid hormone receptor or the glucagon receptor, showed cDNA-dependent constitutive activity with PTH-1 and glucagon. In another functional system, *Xenopus laevi* melanophores transfected with cDNA for the human calcitonin receptor showed constitutive activity. This assay system would show a decrease in the transmittance of light through melanophores when Gs is activated and increased response to calcitonin. Nine ligands for the calcitonin receptor either increased or decreased the constitutive activity in this assay, suggesting that the constitutive system was a sensitive discriminator of positive and negative ligand efficacy (Chen et al., 1999, 2000). This assay system also provides an approach to the identification of ligands for orphan receptors. The argument for this idea is that different conformations of the receptor protein will display different binding domains for ligands and alter the signaling characteristics of the system (Chen et al., 2000).

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