Scientific Background on the Nobel Prize in Chemistry 2012

STUDIES OF G-PROTEIN–COUPLED RECEPTORS

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G-protein–coupled receptors

The Nobel Prize in Chemistry 2012 is awarded to Brian K. Kobilka and Robert J. Lefkowitz for studies of G-protein–coupled receptors.

G-protein–coupled receptors (GPCRs) form a remarkable modular system that allows transmission of a wide variety of signals over the cell membrane, between cells and over long distances in the body. Today, we understand the molecular mechanism of how these receptors work in intricate detail, in large part because of the studies by Kobilka and Lefkowitz.

Background

Every human cell is surrounded by a plasma membrane, a phospholipid bilayer. The membrane makes it possible for the cell to maintain a specific mix of biochemically active species, while preventing unwanted entry of other substances from the outside environment. For proper function, the biochemical machinery inside a cell needs to be able to receive instructions from the outside.

Changes in hormone levels on the outside of the cell elicit adaptive changes in enzyme activity on the inside. Odour molecules affect cells in the olfactory epithelium and substances in the food influence chemical activities in tastebud cells, which in turn elicit electrical signals that transfer information to the brain.

Indeed, human cells are constantly communicating with each other and the surrounding environment, which requires a molecular framework and a mechanism for transmission of information across the plasma membrane. Moreover, in the body, signal transmission may take place over long distances. To be able to respond promptly, the brain needs rapid information from our senses, for vision, smell, taste and more. Again, this requires a molecular mechanism for transmission of information over the plasma membrane.

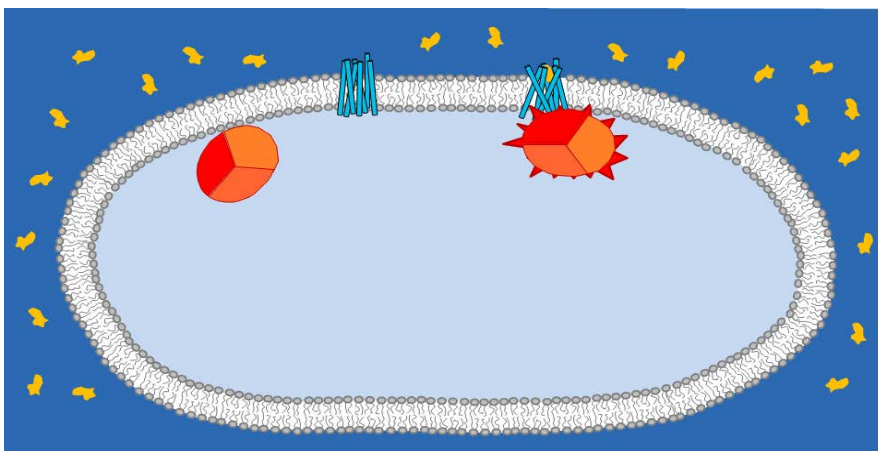

Figure 1. Cartoon of a cell with its interior (light blue) and exterior (blue), with their different chemical compositions separated by a phospholipid bilayer. The bilayer contains many proteins. Shown are two copies of a GPCR with specificity for a diffusible ligand (yellow). The fraction of receptors with bound ligand is governed by the ligand concentration. The receptor to the left is unoccupied and non-activated, and the receptor to the right is occupied by a ligand, bound to a G-protein (red), and activated. The ligand does not pass through the membrane; the signal is transmitted by conformational changes in the receptor protein.

The molecular framework consists of G-protein–coupled receptors (GPCRs). Those are proteins located in the plasma membrane. The name GPCR refers to a common mode of receptor signalling via GTP-binding proteins on the inside of the cell. Because their polypeptide chain passes seven times through the plasma membrane, the GPCRs are also called seven-transmembrane (7TM) receptors. They mediate a wide range of physiological signals from the outside of the cell. The signal can be a change in concentration of peptides, hormones, lipids, neurotransmitters, ions, odourants, tastants, etc., or an influx of photons to the eye. GPCRs convey these signals to the inside of the cell and elicit a series of reactions involving other proteins, nucleotides and metal ions, which eventually deliver a message and an appropriate cellular and physiological response.

Many physiological processes in mammals depend on 7TM receptors, which are also the targets for a large portion of all pharmaceuticals drugs. About a thousand human genes code for 7TM receptors (1,2), and they are involved in sensing a wide range of extracellular stimuli. Examples include the adrenergic receptors, dopamine receptors, histamine receptors, the light receptor rhodopsin, and the many odor and taste receptors.

Key questions

The GPCRs mediate a flow of information that tells the inside of cells about the conditions on their outside. How is this achieved on a molecular level? Which molecules do the job, and how do they send the message? How do they discriminate between different kinds of signals? How are the signals regulated? These have been key questions in the field for decades. To provide answers, researchers needed to identify the molecular components of the signalling machinery and follow their activities in biochemical, biophysical and structural investigations.

Discovery and detection of GPCRs

Most of what we know today about the intriguing molecular properties of 7TM receptors has been discovered over the past 40 years. However, rhodopsin had been identified as a photosensitive pigment as early as the 1870s, and its covalent ligand retinal was reported in 1933 (3). The history of ligand-activated receptors started more than a century ago, when it was noted that reactive cells have a ‘receptive substance’ on their surface (4,5). Early experiments with tissue preparations measured the responses to stimulators (agonists) and inhibitors (antagonists). During the following half century (about 1920–1970), classical receptor theory was developed, based on the law of mass action and dose-response data (6).

Some of the signalling components inside the cell were described in molecular detail before the receptors were. These include the second messenger cyclic AMP (cAMP) and the enzyme adenylyl cyclase (7; Nobel Prize in Physiology or Medicine 1971 to Earl W. Sutherland, Jr.), cAMP-dependent protein kinase (8) and heterotrimeric G-proteins (9-11, Nobel Prize in Physiology or Medicine 1994 to Martin Rodbell and Alfred G. Gilman).

The nature of the ligand-activated receptors remained controversial up to the 1970s. The existence of receptors as separate molecular entities was debated, and it was even speculated that receptors and the enzyme adenylyl cyclase were the same protein.

Chemical synthesis of radioactively labelled agonist ligands became instrumental in proving the existence of receptors and for the detection and visualization of the receptors in the plasma membrane. First to succeed with this approach was Lefkowitz, who modified adrenocorticotropic hormone with radioactive iodine and observed its binding to adrenal membrane preparations (12,13). He then focussed his efforts towards the receptors for epinephrine (also known as adrenaline).

Epinephrine is an important hormone and neurotransmitter that influences many different physiological processes, e.g. regulation of heart rate and blood pressure. Cells responsive to epinephrine have specific plasma membrane receptors (adrenergic receptors) that interact with the hormone. There are at least nine different adrenergic receptors, grouped as α- and β-receptors. Different tissues and organs respond in different manners to elevated epinephrine concentration. A main goal was fulfilled when radioligands specific for the β-receptors were found (14-16). The field exploded, and radioligands with retained specificity were developed for a large number of receptors. Radioligands were also used to quantify and compare the effect of a range of adrenergic compounds on the activity of β-adrenergic receptors and adenylyl cyclase. Studies of the thermodynamic coupling between ligand- and G-protein–binding events provided insights into the signalling mechanism (17,18).

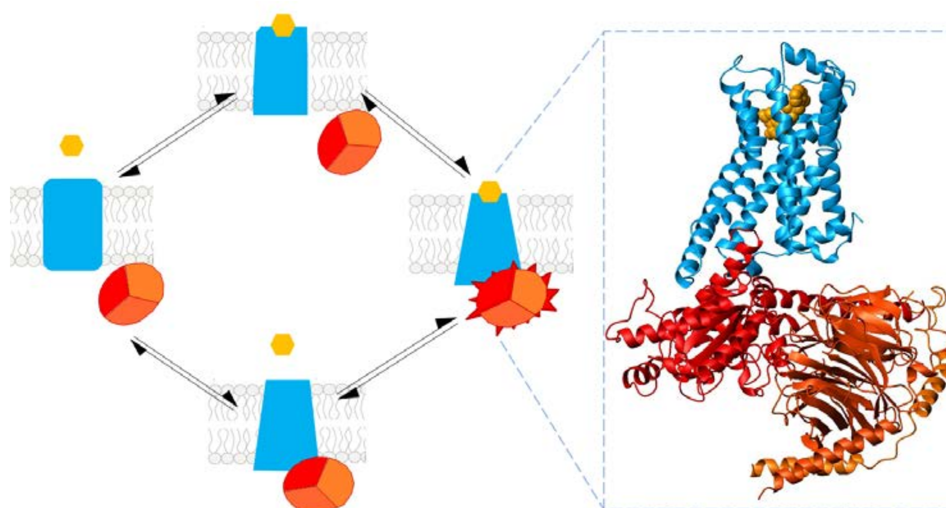

Figure 2. Left: Ternary complex model adapted from (17). A thermodynamic cycle describes the formation of a complex of ligand (yellow), receptor (blue) and G-protein (red). **Right:** Crystal structure of an active ternary complex. Ribbon model drawn from the coordinates from file 3sn6.pdb (19) using the software Molmol (20).

The ternary complex model

In 1980, a general mechanism for receptor activation, the so-called ternary complex model (17), was proposed by Lefkowitz and his coworkers Andre De Lean and Jeffery Stadel. The ternary complex of extracellular ligand (agonist), transmembrane GPCR and intracellular

G-protein serves as an activated signalling unit (17). The agonist (hormone, neurotransmitter, odourant, etc.) binds to the extracellular part of the receptor, while G-protein binds to the intracellular regions (Figure 2).

Receptor signalling relies on an allosteric mechanism, and the allosteric coupling is mutual, as follows from the laws of thermodynamics; binding of agonist increases the affinity for the G-protein on the inside of the cell, and binding of G-protein increases the receptor's affinity for agonist binding to the same extent (17). After three decades of dedicated research, we understand the mechanism for trans-membrane signalling by GPCRs and their regulation in molecular detail. In a crowning achievement, Kobilka and his coworkers revealed the three-dimensional structure of a fully functional ternary complex at high resolution: the β2-adrenergic receptor (βAR) in complex with agonist and G-protein (19).

Isolation and purification

Isolation and purification of GPCRs in functional form in vesicles (spherical model membranes) was first achieved for rhodopsin by Ruth Hubbard (21). Receptors activated by diffusible ligands are generally found in very low amounts, for example in tissues like liver, lung and heart, and their isolation is therefore challenging. Reports on solubilized receptors for vasopressin, thyrotropin and parathyroid hormone came in 1975 (22-24). The choice of detergent proved to be the key for the solubilization of a functional βAR by Marc Caron and Lefkowitz (25,26). They used the same detergent as used in the earlier isolation of functional rhodopsin (21), an intriguing coincidence considering that the sequence homology between the two was unknown at the time. Affinity chromatography methods were developed to purify the receptors using specific ligands conjugated to chromatography resins (27-29). With a few additional steps, β-adrenergic receptors were purified to near theoretical specific activity (30-32).

A family of structurally related receptors

Lefkowitz and coworkers made a seminal contribution when they cloned and sequenced the first receptor for epinephrine, βAR (33). Based on this work, it became clear that GPCRs form a family of proteins with a close structural relationship. Although the signals picked up by GPCRs differ widely – ranging from photons and odourant to neurotransmitters, hormones and peptides – the transmission of the signal into the cell is accomplished in a highly similar manner by a common structural framework of seven transmembrane helices.

Lefkowitz and his coworkers spent more than a decade of dedicated work to find suitable ligands for use in affinity chromatography. They learned how to purify and handle βAR. They isolated sufficient amounts of this low-abundance membrane protein from hamster lung for N-terminal amino acid sequencing of discrete peptides. Based on this information, they constructed degenerate oligonucleotides and used those for cloning of the βAR gene. Kobilka, then a young post-doc with Lefkowitz, came to play a key role, and he is acknowledged by Lefkowitz for envisaging and successfully constructing a genomic DNA library superior to the cDNA library that was previously available for the cloning work.

Fortunately, the gene has no introns (34) and the entire sequence could be derived. This was a major breakthrough that revealed an astonishing surprise: the presence of the seven transmembrane helices, plus homology to the rhodopsin sequence (35-37) within several of the transmembrane spans (33). The researchers inferred that all G-protein–coupled receptors share this structural arrangement (33). This was corroborated by Shosaku Numa, who presented the sequence of a third GPCR: the muscarinic acethylcholine receptor (38). Lefkowitz and his colleagues cloned a series of related adrenergic receptors. βAR and rhodopsin became the founding members of a huge receptor family and the basis for all future work in this field (39,40).

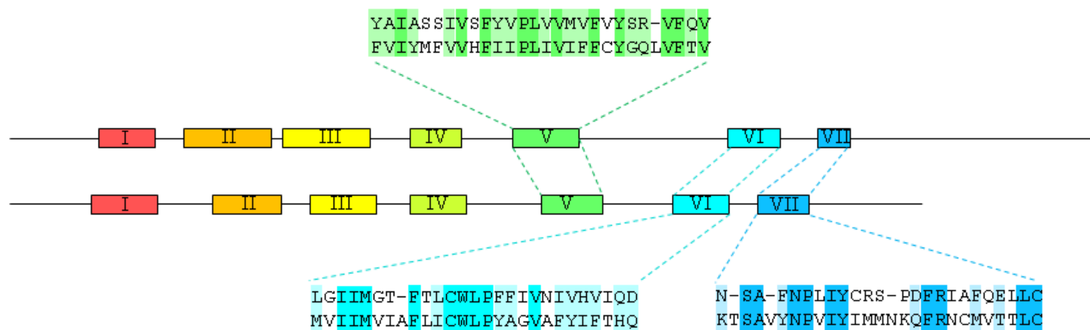

Figure 3. The prediction of seven helices in βAR is shown above rhodopsin. The homologous amino acid sequences in helices five, six and seven are aligned, and identities and similarities are coloured. Adapted from reference 33.

Signalling mechanism

An important aspect of the signalling mechanism is that the ligand does not pass through the membrane. Instead, the signal is transferred to the inside of the cell by conformational changes in the receptor protein, which are coupled to ligand-binding events. An increase in concentration of agonist on the outside of the cell increases the fraction of receptors that are bound to a ligand.

The receptor protein in the membrane is dynamic and can take on a number of conformations; two prominent ones are called the inactive and active conformation. Each receptor has high affinity for a small set of similar agonists that bind to a specific site in its extracellular part. Binding of agonist favours the active receptor conformation, and increases the affinity for the G-protein on the inside of the cell.

The cascade reaction on the inside of the cell starts with nucleotide exchange and the G-protein dissociates into subunits (Gα, Gβ and Gγ; 41). Gα binds to and stimulates enzymes such as adelnylate cyclase. This produces the cyclic nucleotide cAMP, which diffuses easily and serves as a ‘second messenger’. Other proteins may interact with Gβ and Gγ to further modulate the signal. The activated GPCR conformation lasts long enough to allow one bound agonist molecule, or one adsorbed photon, to activate several G-proteins, which amplifies the signal (42,43). Reacted G-proteins reassociate after nucleotide hydrolysis and may reenter the cycle.

The first insights into the structural changes during signalling were derived from biochemical and biophysical experiments. Wayne Hubbell and Gobind Khorana found that motions of helix 6 are important in the activation mechanism, by using electron paramagnetic resonance (EPR) spectroscopy and spin-labeled cysteine mutants (44). Further details of the structural transitions emerged from studies using fluorescence, infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, covalent cross-linking and mass spectrometry in combination with amide proton exchange (45-53). The results of these studies provide further details into the structural transitions between the end states, the free receptor versus ternary complex. There are distinct structural changes around the bound agonist and extensive reorientation of the transmembrane helices.

As in other proteins that transmit information over a distance, a helical framework amplifies a small conformational change at one end of the protein into a much larger conformational change at the other end. Helices are rod-like structures, and one might imagine a 7TM receptor as a bundle of rods immersed in the membrane. If a ligand grips the bundle at one end, the bundle opens up like a bouquet of roses at the other end. Small changes in the network of side-chain interactions around the agonist-binding site propagate into larger structural changes at the intracellular side. This opens up a binding site for the G-protein and constitutes a signal sent from the outside to the inside of the cell.

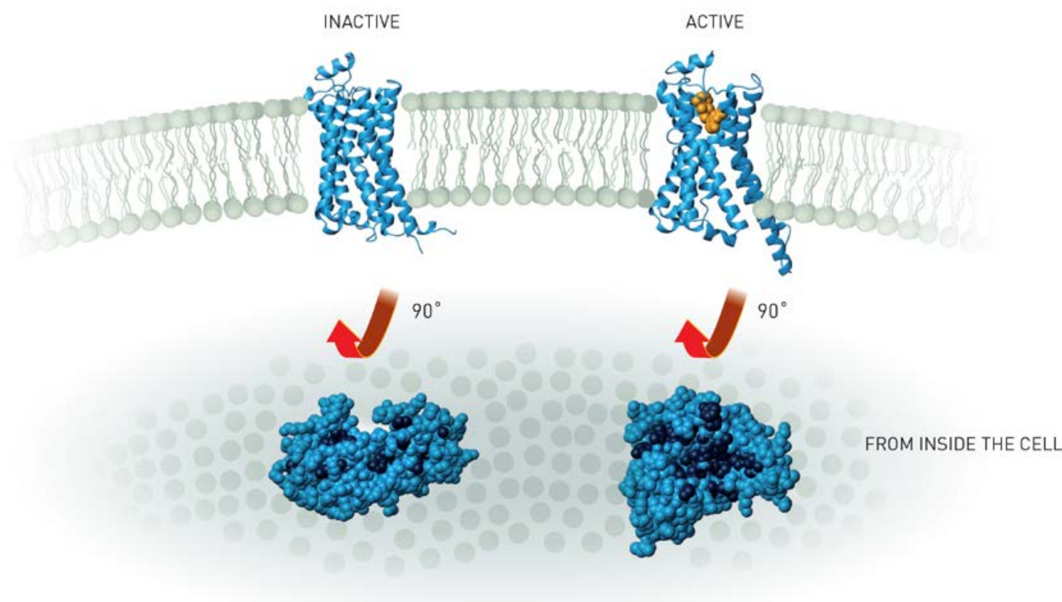

Figure 4. Structural basis of the GPCR signalling mechanism. Non-activated βAR (2bar.pdb) is shown to the left, and activated βAR bound to ligand and G-protein (3sn6.pdb) to the right. At the top, the receptor in the membrane is drawn with blue ribbon that traces the backbone. The bottom view is from the inside of the cell membrane, with the receptor shown using a space-filling model with hydrophobic side chains in dark blue.

The Holy Grail, a high-resolution structure of an active ternary complex, was recently found by Kobilka and coworkers (19). This structure of βAR in complex with heterotrimeric G-protein and agonist ligand provides the most detailed insights into the structural transition

underlying the activation mechanism through comparison with the non-activated receptor (54-56). The small structural change around the ligand propagates into a much larger structural transition on the inside than previously anticipated. There is a large displacement of helix 6 and an opening of a deep hydrophobic cleft on the intracellular side of the receptor (Figure 4). The C-terminal helix of Gα penetrates into this cleft and this leads to large structural changes in the G-protein, which is thereby activated. The structural changes result from the rearrangement of a network of interactions in the receptor.

The ability to construct a relatively stable ternary complex was the result of sophisticated molecular biology developments (19). The inherent instability of the complex between receptor and G-protein presented a major challenge. The complex is short-lived, enabling many turnovers of G-protein activation for each ligand-binding event.

A combination of five biochemical strategies produced a long-lived ternary complex suitable for crystallization: apyrase treatment of the complex with GDP-bound G-protein; stabilization of the complex using a newly developed detergent; selection of a camelid antibody, a ‘nanobody’, that stabilized the Gβ-subunit in a well-defined conformation; covalent agonist coupling; and cloning of T4-lysozyme into the loop between helix 5 and 6 to reduce its mobility. Isotope exchange studies of the unmodified complex combined with mass spectrometry show that the structure of the receptor in the ternary complex is essentially the same as in an unmodified complex (57).

Earlier structures

The first view of a 7TM receptor was the projection structure of rhodopsin found by Gebhard Schertler and Richard Henderson using cryo-electron microscopy (58,59), showing the seven α-helices in the membrane. To achieve higher resolution and three-dimensional structures, Tetsuji Okada and coworkers developed a purification strategy (60), and this led to well-diffracting crystals (61). Krzysztof Palczewski and Okada solved the three-dimensional structure of non-activated rhodopsin (62,63), providing a more detailed view of the helices in the membrane and the loops, some of which fold over the bound retinal.

Crystallographic studies of ligand-activated GPCRs in the absence of agonist were challenging because of significant receptor mobility. Kobilka responded to this challenge together with Gebhard Schertler (54) and Raymond Stevens (55,56*)* using antibodies, cloning and addition of cholesterol. In this structure, the receptor helices are more parallel compared to the ternary complex, and the intracellular part of the receptor does not present any large hydrophobic surface (Figure 4). The non-activated βAR structure differs from rhodopsin in the ligand-binding site and other regions with weaker interactions between the cytoplasmic ends of helix 3 and 6.

Klaus Peter Hofmann presented crystal structures of opsin (64) and opsin with a bound peptide fragment from the G-protein transducin (65). These structures of rhodopsin without retinal ligand represent a partially activated receptor, with a shifted tilt and rotation of helix 6 and with binding of the G-peptide in a cleft on the intracellular side.

Kobilka solved the crystal structure of agonist-coupled βAR in complex with a nanobody as a surrogate for the G-protein (66). The structural transitions relative to inactive βAR are remarkably similar to the structural changes in opsin relative to rhodopsin. These findings eradicated the artificial division between light- and ligand-activated receptors, and completed the avenue opened in 1986 by the notion of a common structural framework in GPCRs (33). Insights derived from rhodopsin have deepened the understanding of ligand-activated receptors, and *vice versa*.

Pharmacological value of agonists, antagonists and inverse agonists

Many small molecules serve as agonists, inverse agonists and antagonists that modulate cellular activity by binding to GPCRs in different manners to elicit specific responses. The term agonist is reserved for ligands that bind to a GPCR and stabilize a conformation that activates the G-protein on the inside. A substance that binds to and stabilizes the inactive form of the receptor is called an inverse agonist. Antagonists or inhibitors compete with the agonist and bind to and block the agonist-binding site. Antagonists thereby inhibit GPCR-mediated signalling by preventing the conformational change that activates the G-protein. This is the basis for extensive pharmaceutical development and makes GPCRs very important drug targets.

Inhibitors are of great pharmacological value, and Sir James W. Black shared the Nobel Prize in Physiology or Medicine 1988 for his discovery of propranolol and cimetidine, which inhibit the βAR and the H2 histamine receptor, respectively. Propanolol and its derivatives are β-blockers used in the treatment of conditions involving the heart and elevated blood pressure. Other pharmaceutical compounds are agonists, which activate, for example, the dopamine and serotonin receptors to alleviate Parkinson's disease, migraine and neuropsychiatric conditions, or they are inverse agonists, which prevent basal activity of, for example, the GABA receptor involved in memory and learning.

The ternary complex structure (19) and a range of other structures provide a basis for pharmacologic development of drugs with high specificity, efficacy and few side effects. The biochemical strategies developed by Kobilka for βAR make it possible to produce crystals of other 7TM receptors as well. These techniques have already been adopted by other groups in order to produce crystals and solve high-resolution structures for a number of pharmacologically important receptors.

Modular system and G-protein–independent signalling

GPCR signalling is an elegant modular system with components that are used over and over again for many kinds of signals. The same receptor can also signal through different intracellular pathways depending on the identity of the bound ligand. This is due to the relative flexibility of the receptor in the membrane, which allows different agonists or inverse agonists to stabilize different active or inactive forms.

Lefkowitz and coworkers found G-protein–independent pathways (67), in which the 7TM receptors signal to the inside of the cell via other proteins, including arrestins. Therefore,

'7TM' rather than 'GPCR' is becoming the preferred label for this receptor family. The signalling pathway is selected based on ligand identity, and the same 7TM receptor may be involved in both G-protein–dependent and G-protein–independent signalling (68,69). In current terminology, this is referred to as 'biased ligands'. The small structural transition on the extracellular side of the receptor is important for the ability of biased ligands to select an activation pathway. Insights into this phenomenon and its structural basis will improve the pharmacological specificity and pathway selectivity of drugs targeting 7TM receptors (70).

The modular design of 7TM signalling is used even more widely by nature, for example with respect to a specific ligand. The nine adrenergic receptors allow a single physiological signal of enhanced epinephrine concentration in blood to cause diverse effects in different organs. While epinephrine binding to the receptors in heart and lung leads to activation, binding to the receptor in the gut leads to downregulation of digestion. Biochemical studies of chimeric adrenergic receptors, built from parts of an activating receptor and parts from a downregulating receptor, have defined the parts of the receptor responsible for ligand binding and G-protein activation, respectively. This has deepened our molecular level understanding of this diversity in signalling mechanism (71).

Covalent versus noncovalent ligand

Rhodopsin has a central role in vision. It differs from many other 7TM receptors in that its retinal ligand is covalently bound. Therefore, signalling becomes independent of ligand concentration, and this allows rhodopsin to respond to another type of signal: the influx of light. Adsorption of a photon shifts the conformation of retinal from the *cis*- to the *trans*-isomer, which causes a conformational change of rhodopsin (52). The active conformation is favoured by *trans*-retinal, while *cis*-retinal stabilizes the inactive form. Inside the cell, rhodopsin functions via a G-protein (transducin) phosphodiesterase and cyclic-GMP, resembling the βAR receptor signalling via G-protein, adelnylate cyclase and cAMP (72-76).

Regulation of 7TM receptors

There must be mechanisms for the deactivation and regulation of the signal from an active GPCR, or the signal becomes permanent. A fundamental question is, therefore, what molecular mechanisms regulate and terminate the signal?

Biochemical studies during the 1970s and 1980s provided several answers. The signal can be interrupted by dissociation of the agonist, but it can also be terminated or downregulated through biochemical regulation of the receptor. Exposure to ligand may lead to downregulation (77). Phosphorylation of the intracellular part makes the receptor less sensitive to the ligand concentration (78) or light (79); in other words, the receptor becomes desensitized. Specific enzymes conduct this chemical modification, for example, the β-adrenergic receptor kinases (80) and rhodopsin kinase (81). Phosphorylation increases the affinity of the receptor for regulatory proteins called arrestins (82,83), leading to further downregulation. Kobilka found that internalization via endocytosis and recycling of receptors are other mechanisms for regulation of receptor signalling (84,85).

In some genetic diseases, mutations cause basal receptor activity. Studies of these mutants by Lefkowitz provide insights into which intramolecular interactions keep the normal receptor inactive in the absence of bound agonist (86). At one adrenergic receptor position, he found that all other 19 amino acids except the native one lead to basal activity (87). Receptor activity might also be modulated by the association of receptors in the membrane to form homo- or heterodimers or higher order assemblies (88).

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Nobel Prizes in Physiology or Medicine in the area:

1967. Ragnar Granit, Haldan Keffer Hartline and George Wald for the physiological and chemical processes underlying photoreception.
1971. Earl W. Sutherland, Jr., for cyclic AMP (cAMP)
1988. Sir James W. Black for the discovery of propranolol, which blocks the β-adrenergic receptor, and the H2 histamin receptor blocker cimetidine.
1994. Martin Rodbell and Alfred G. Gilman for heterotrimeric G-proteins
2004. Linda B. Buck and Richard Axel for odourant receptors.

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