The G Protein-Coupled Receptors Handbook

Contemporary *Clinical* Neuroscience

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Contemporary Clinical Neuroscience

The G Protein-Coupled Receptors Handbook

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Cover illustration: Atomic force microscopy (AFM) of native mouse disk membranes imaged in buffer solution (Fotiadis, D., et al. [2003] Nature 421:127). Based on the AFM data and crystal structure of rhodopsin (Palczewski, K., et al. (2000). Science 289, 739), a 3-D model for the packing arrangement of rhodopsin molecules within the paracrystalline arrays in native disk membranes as computed and 6 rhodopsin molecules are displayed (Liang, Y. et al. [2003] J Biol Chem 278:21,655). Artwork created by and courtesy of Kris Palczewski, Andreas Engel, and Slawek Filipek.

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FOREWORD

Comprising the largest class of membrane-bound receptors, the G protein-coupled receptors (GPCRs) also represent one of the most prevalent gene families. These receptors mediate the biological effects of numerous hormones, neurotransmitters, chemokines, odorants, and other sensory stimuli. These in turn control such diverse physiological processes as neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, and inflammatory and immune responses. In short, GPCRs are involved in a myriad of processes in the human body relevant to health and disease. Consequently, the GPCRs are targets of approx 70% of pharmacological therapeutics and provide further important opportunities for the development of new drug candidates with potential applications in all clinical fields. Recent progress in GPCR research has proliferated at a remarkable rate.

Structurally, GPCRs are characterized by a seven-transmembrane a-helical (7TM) configuration of more than 25% homology, but detailed structural knowledge is sparse. Of the three distant families of vertebrate GPCRs, family A is by far the largest group, and includes rhodopsin, adrenergic receptors, and the olfactory subgroups. The receptors for the gastrointestinal peptide hormone family belong to family B, whereas family C includes the metabotropic glutamate/pheromone receptors. The recent availability of the structure of rhodopsin has given a basis to better understand structure–function relationships in other GPCRs. Subsequently, sequence-based predictions and molecular modeling incorporating a multitude of results from biochemical and biophysical analyses can now be scrutinized, demonstrating some degree of success for these methods. Despite the progress made in predicting the critical residues engaged in ligand binding, particularly within the large family A, detailed structural knowledge is still required for understanding the process of signal transduction at a mechanistic level. Current work focuses on determining the structure of other GPCRs, on elucidation of their interactions with ligands, and on conformational changes during their activation process. There is significant hope that additional breakthroughs will occur in the near future.

The classical view that GPCRs function as monomeric entities has been jarred by the emerging concept of GPCR dimerization. Examples of GPCRs that can be biochemically detected in homo- or heteromeric complexes are being reported at an accelerated rate. These findings have not only indicated that many GPCRs exist as homodimers and heterodimers, but also that their oligomeric assemblies could have important functional roles. The important observation of GPCR dimerization came through the direct visualization of rhodopsin dimers in native disk membranes by atomic force microscopy. The ability of GPCRs to specifically oligomerize may provide some insight into how different receptor pathways influence each other. The general acceptance of the existence of GPCR dimers is now likely to have important implications for the development and screening of a new class of drugs.

The G Protein-Coupled Receptors Handbook gives a broad overview on the most recent progress in the rapidly evolving field of GPCR research. It comes at a timely period because of the significant advances that have been made in the last few years in the understanding of the structure and function of GPCRs.

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PREFACE

The intent of *The G Protein-Coupled Receptors Handbook* is to provide a comprehensive overview of recent advances in the G protein-coupled receptor (GPCR) field. From the basics of GPCR structure to dimerization and drug discovery, this book reviews much of the recent advances and current knowledge regarding GPCRs.

The first few chapters focus on the fundamentals of GPCR structure and function. GPCR function is now known to be regulated by a number of mechanisms: ligand-induced conformational changes, stabilizing intramolecular interactions, pharmacological chaperones, and membrane trafficking all play a role in regulating GPCRs. Specific ligand binding causes changes in GPCR conformation, which ultimately result in the activation of intracellular signaling cascades. Meanwhile, the inactive state of the receptor is maintained by stabilizing intramolecular interactions; disruption of these interactions is necessary for receptor activation. Pharmacological chaperones play a role in GPCR folding and maturation, and appear to be involved in a number of human genetic diseases. Finally, membrane trafficking of GPCRs in endocytic and biosynthetic pathways also contribute to the physiological regulation of GPCRs.

GPCRs are present in every cell and interact with a multitude of downstream effectors: heterotrimeric G proteins, regulators of G protein signaling (RGS), arrestins, G protein-coupled receptor kinases (GRKs), and many other GPCR interacting proteins. Heterotrimeric G proteins are among the most important signaling transducers involved in GPCR activity, directly coupling to the receptor and transmitting its information about activation/inactivation to the cell. RGS proteins are involved in the regulation and termination of the signaling process. GRKs catalyze GPCR phosphorylation, promoting receptor desensitization and internalization. Arrestins mediate the desensitization and uncoupling of GPCRs from their G proteins, and may also function as signal transducers. In addition, β-arrestin regulates the sequestration, intracellular trafficking, degradation, and recycling of most GPCRs. More than 50 other GPCR interacting proteins have been identified that function as modulators of GPCR function at various stages of signaling.

The next section of this book explores our current understanding of GPCR dimerization. The emerging concept of dimerization has modified our views of GPCR structure, function, and regulation tremendously. The existence of GPCR dimers has been demonstrated using biochemical

methods, such as co-immunoprecipitation, and biophysical approaches, such as fluorescence (FRET) and bioluminescence resonance energy transfer (BRET). Potential domains of GPCR dimerization have been described using computational and experimental approaches. Functional complementation studies have been used to analyze the basis, selectivity, and mechanisms of dimerization. It is now evident that dimerization plays a role in receptor maturation, as many GPCRs have been shown to dimerize prior to their trafficking to the cell surface. There is also some evidence suggesting that dimerization alters the endocytotic and postendocytotic trafficking properties of GPCRs. More importantly, heterodimerization has been shown to modify the pharmacological properties of GPCRs; a finding that could have an enormous impact on the future of drug design.

The final chapters of this book describe some of the most recent developments in the GPCR field, leading to advances in drug discovery. It is now thought that a number of GPCRs functionally interact as heterodimers to mediate analgesic responses. Elucidating the role of GPCRs in mediating pain is also crucial to the development of superior analgesic drugs. Thus, a new wave of drugs specifically targeting heterodimeric receptor complexes may be on the horizon. Another important area of current research consists of investigating the structural plasticity of receptor activation by examining the conserved motifs contributing to the overall receptor structure (and variability among subtypes); this would confer ligand-binding specificity and, thus, could lead to the development of receptor-type selective drugs. Finally, the last chapter describes the identification of natural ligands of orphan GPCRs, i.e., deorphanization. Orphan receptors may represent an untapped drug target. Understanding the evolutionary diversity in GPCR ligand recognition is fundamental to understanding the potential of GPCRs as therapeutic targets.

I thank all the authors for their timely and insightful contributions, the series editors Helen Baghdoyan and Ralph Lydic for suggesting this book as a part of their series, and Ms. Elyse O'Grady at Humana Press for keeping things moving along. I also thank Noura Abul-Husn, Fabien DeCaillot, and José Morón for their extensive input into the chapters. Finally, I am grateful to Dr. Ivone Gomes for her excellent assistance throughout all of the editing and formatting stages. From planning the list of chapters/authors to the realization of this book, it has been a rewarding experience; I hope this book will serve as a helpful guide for those who are interested in learning more about the function and regulation of GPCRs.

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Color plates 1–7 appear in an insert following p. 368.

I

GPCRs: Structure and Function

1

Structure–Function Relationships in G Protein-Coupled Receptors

Ligand Binding and Receptor Activation

Dominique Massotte and Brigitte L. Kieffer

1. INTRODUCTION

G protein-coupled receptors (GPCRs) are integral membrane proteins that form the fourth largest superfamily in the human genome, with more than 800 genes identified to date *(1,2)*. Many of these receptors play key physiological roles, and several pathologies have been associated with receptor functional abnormalities *(3,4)*. Therefore, GPCRs represent important targets for drug design within pharmaceutical companies *(5)*. Indeed, GPCRs mediate the effect of numerous ligands, including neurotransmitters, chemo-attractants, hormones, cytokines, and sensory stimuli such as photons and odorants.

GPCRs were named for their common ability to associate with heterotrimeric G proteins (Gαβγ). Binding of extracellular ligands with agonistic properties initiates the signal transduction cascade by triggering conformational changes in the receptor that promote heterotrimeric G protein activation *(6,7)*. Following nucleotide exchange (guanosine diphosphate [GDP] replacement by guanosine triphosphate [GTP]), the tightly associated Gα and Gβγ-subunits separate from each other and from the receptor. Both components are then free to interact and modulate the activity of downstream elements of the signaling cascades, such as adenylyl cyclase, phospholipases, mitogen-activated protein kinases (MAPKs), or calcium and potassium ion channels. Signal transduction is tightly regulated by receptor posttranslational modifications. Among them, receptor phosphorylation by GPCR-specific and -nonspecific kinases modulates subsequent interactions with several intracellular proteins involved in receptor internalization and downregulation *(8,9)* or promoting growth factor receptor transactivation

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(10). Additional regulatory mechanisms ensue from the interplay of G protein subunits with regulators of G protein signaling (RGS) *(11)*.

Tremendous progress has been accomplished within the past few years in dissecting GPCR-mediated signal transduction pathways, but the molecular mechanisms underlying ligand recognition and signal transduction through the membrane are restrained by the lack of detailed receptor structures. To date, only the three-dimensional (3D) structure of rhodopsin has been solved at high resolution *(12)* because of the difficulty in producing large amounts of concentrated integral membrane proteins, even in heterologous expression systems *(13)*. Moreover, purification of GPCRs retaining structural integrity requires defined compositions and ratios of lipids and detergents. Additionally, GPCR size is fairly large (from approx 40 kDa to 200 kDa), which further hampers their study. Altogether, these distinctive features have prevented the acquisition of 3D structural information by means of crystallography as well as nuclear magnetic resonance (NMR) techniques. Thus, most of the structural information gathered to date derives from mutagenesis studies or biochemical and biophysical approaches, to which models based on the rhodopsin structure are now added. Our view depicts GPCRs as a bundle of seven-transmembrane α-helices alternatively connected through intracellular and extracellular loops. The N-terminal part of the receptor is located on the extracellular side of the cytoplasmic membrane, whereas its C-terminal counterpart faces the cytoplasm.

2. GPCR CLASSIFICATION

Few sequences are conserved among the GPCR superfamily, which is often divided into six classes (*see* GPCR Database available at http://gpcr.org/). Distinctive structural elements that characterize the three main GPCR families (A, B, and C) are summarized in Fig. 1.

Class A receptors, also called rhodopsin-like receptors, comprise the largest family of GPCRs. This class of receptors binds ligands from various types, including small molecules such as biogenic amines as well as peptides (*see* Subheading 4.). The overall homology among all class A receptors is restricted to a limited number of highly conserved key residues in the transmembrane regions, suggesting a critical role in the structural or functional integrity of the receptor. Ligand binding to class A receptors is discussed in detail in Subheading 4.

Class B receptors, also called secretin-like receptors, include about 20 different receptors for various hormones and neuropeptides *(2)*. Ligand binding involves both the N-terminus and extracellular loops of the receptor, and to date, no evidence has been obtained regarding interactions occurring within the transmembrane region of these receptors.

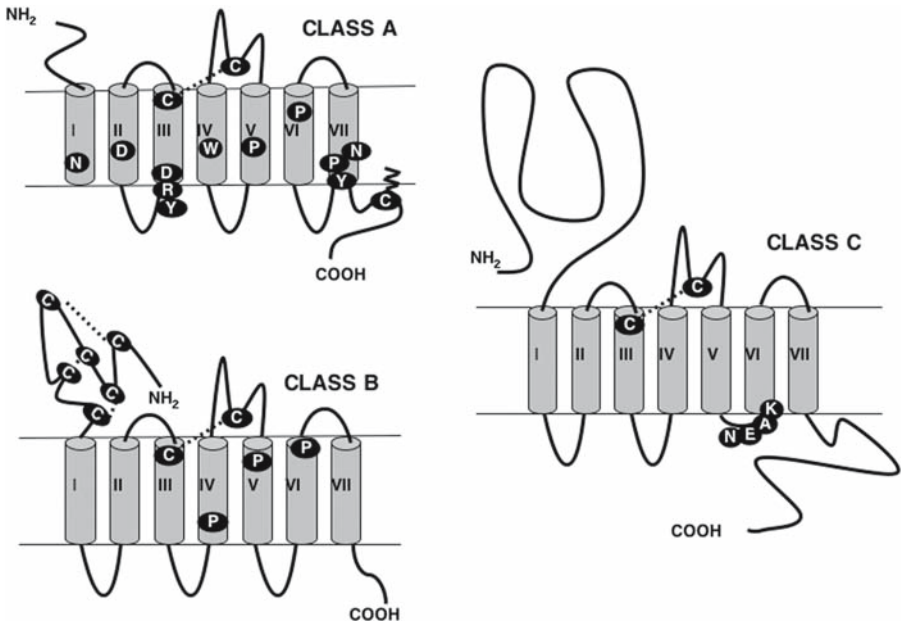

Fig. 1. The G protein-coupled receptor (GPCR) main families. A schematic representation is shown for the three main GPCR classes (A, B, and C) with common structural motifs to each family. The only common motif to class A, B, or C GPCRs is a conserved cysteine residue on helix III and another in the second extracellular loop 2. These cysteines are believed to be connected via a disulfide bridge. For class A receptors (rhodopsin-like family), the most conserved amino acid of each helix is indicated. A putative palmitoylation site is represented in the proximal part of the C-terminus (≈). The DRY motif on helix III and the NPXXY motif on helix VII are conserved among class A GPCRs (see details in Fig. 2A). Class B GPCRs (secretin-like family) share a large amino terminus with conserved cysteine residues and disulfide bridges. Some proline residues are also conserved within the helical bundle, but those residues are different from class A conserved prolines. Class C receptors (metabotropic glutamate family) are characterized by a very large extracellular domain that binds the ligands. The highly conserved motif NEAK (NDSK in the case of the GABA family) on the very short intracellular loop 3 is indicated.

In class C receptors, also known as metabotropic glutamate receptors, ligand recognition is achieved through their very large extracellular domain (300–600 residues). It is organized into two distinct lobes separated by a cavity that binds the ligand in a "Venus flytrap" manner *(14)*.

Classes D and E constitute two minor families that are present in fungi and recently the frizzled/smoothened receptor family was added to the world of GPCRs *(15)*.

3. OVERALL TOPOGRAPHY OF CLASS A RECEPTORS

Despite limited sequence homology, class A receptors exhibit identical structural organization, and their overall topography can be subdivided into three main regions (Fig. 2A).

On the extracellular side, the N-terminal region is involved in ligand binding (*see* Subheading 4.) and possibly receptor activation (*see* Subheading 5.2.), whereas the extracellular loops represent important key elements for peptide binding and play a role in receptor selectivity toward ligands (*see* Subheading 4.3.).

The transmembrane core is comprised of a bundle of seven α-helices that provide a hydrophobic environment critical for nonpeptide as well as small-peptide ligand binding (*see* Subheadings 4.1. and 4.2.; Fig. 2B). It relays the conformational changes induced upon ligand binding on the extracellular side of the receptor to the intracellular architectural determinants that regulate activation of the signaling cascade (*see* Subheading 5.2.).

On the intracellular side, the loop regions contain key elements for either direct or scaffolding–protein-dependent interactions with intracellular effectors (*see* Subheadings 5.4. and 5.5.). Additionally, posttranslational modifications present in the C-terminal are likely to modulate both receptor activation state and G protein coupling (*see* Subheading 5.3.) as well as to participate in the regulation of receptor internalization and desensitization.

4. LIGAND BINDING IN CLASS A RECEPTORS

Countless studies have been performed on individual receptors that now allow us to draw a fairly consistent picture of the precepts that govern ligand binding to class A receptors. Information on critical determinants has been experimentally obtained using site-directed as well as random mutagenesis, receptor chimeras, and biochemical and biophysical methods. Such experimental data were combined with computer modeling and were used to refine the proposed models. This approach led to an improved template that has been used for ligand-docking studies *(3,16)*. One major outcome was the notion that both the size and nature of the ligand drastically influence the modalities and location of its binding. Hence, only some commonalities may be extracted that are general among ligands or receptors.

4.1. Binding of Small Ligands Within the Receptor Transmembrane Regions

On the basis of the crystallographical information obtained for rhodopsin, Ballesteros et al. *(17)* performed a detailed structural comparison of the D2 dopamine receptor with rhodopsin and concluded that the rhodopsin and biogenic amine receptors may be very similar, despite structural divergence in the transmembrane helical bundle. Indeed, helix kinks at proline (Pro) residues or helix binding or twisting at cysteine, serine, or threonine residues may slightly modify the shape of the ligand-binding pocket and introduce the subtle differences required for class A receptors to bind a structurally diverse collection of ligands. Conserved Pro-kinks in helices V,VI, and VII could adopt different conformations that could significantly change the binding sites of different GPCRs. Nonconserved Pro residues in helices II and IV or nonconserved cysteine/threonine/serine residues in helix III and other helices are another source of potential structural divergence in the binding-site crevice. The authors postulated that GPCRs have evolved in a way that maintains their overall fold by means of alternative molecular mechanisms (structural mimicry) that enable localized variations within their binding sites suitable for recognizing a wide variety of ligands. As a consequence, if the crystal structure of rhodopsin can be used as a template for class A receptor modeling, the particular conformation of the binding site of a given receptor may require substantial refinement to be accurately described at the molecular level.

However, some structural elements represent a very specific signature for a receptor family. Catecholamines and related biogenic amines bind primarily within the transmembrane region of their receptors. The identified binding crevice is outlined by residues from helices III, V,VI, and VII. This binding pocket is common to both agonists and antagonists that likely establish a salt bridge with a conserved aspartate residue on helix III at a position analogous to D113 (3.08)[1] in the β2-adrenergic receptor (AR). Additional key interactions have also been identified that differ between agonists and antagonists *(6,18)*.

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[1]Amino acids will be referred throughout the text according to the one-letter code. Residue numbering in parentheses corresponds to the nomenclature introduced by Ballesteros and Weinstein for amino acids located in the transmembrane region of the receptor. The first number refers to the helix on which the residue is located. The second number indicates the position of the residue relative to the most conserved amino acid on this helix to which an arbitrary value of 50 is assigned. Residue 3.44 for example is located on helix III six amino acids before the conserved arginine.

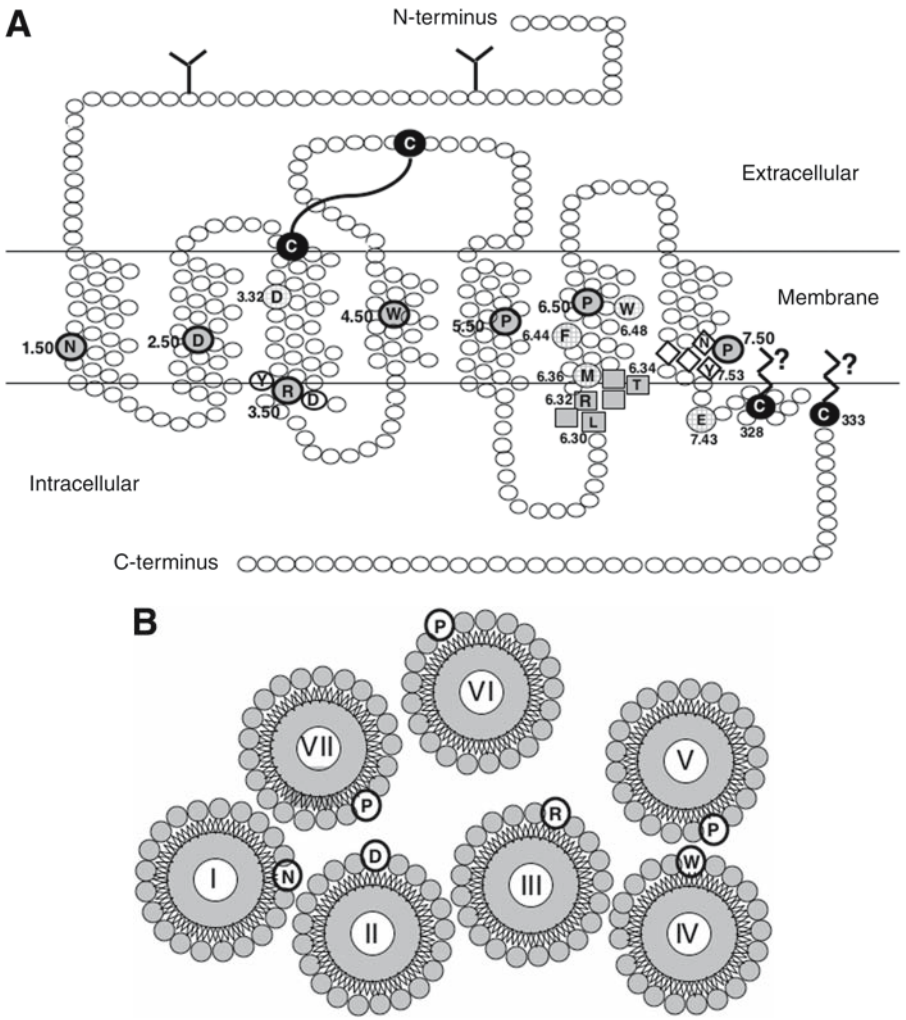

Fig. 2. Schematic representation of class A receptors. (**A**) General organization of the δ-opioid receptor as a prototype for class A receptors. The amino terminus is located at the extracellular side of the membrane and the carboxy-terminus at the cytoplasmic side. *N*-glycosylation consensus sequences are present (Y) on the N-terminus. The conserved cysteines forming a disulfide bridge between helix III and the extracellular loop 2 are indicated. Potential palmitoylation sites at the proximal part of the receptor are represented (≷). Residues in the transmembrane region are numbered according to the nomenclature of Ballesteros–Weinstein. Highly conserved residues among class A G protein-coupled receptors (GPCRs) are indicated by a grey circle with a thick black border. Important motifs are also located. Residues of the E/DRY motif on helix III are shown in white circles with a thick border. Residues forming the basic $X_1BBX_2X_3B$ motif on helix VI are indicated by gray

Based on the mutagenesis studies performed on GPCRs that bind cationic amine neurotransmitters, the aspartate residue in helix III has become a systematic anchorage point for amine ligands in modeling studies. However, opposite effects were observed when this assumption was tested on the μ- and δ-opioid receptors, leading to the conclusion that the aspartate residue in helix III is required for high-affinity binding of agonists to the μ-opioid receptor, but not the δ-opioid receptor *(19,20)*. Moreover, extensive mutagenesis studies performed on the δ-opioid receptors involving peptides and alkaloids acting as agonists as well as antagonists emphasized that the determinants of the opioid receptor binding pocket differ among ligands, despite the presence of a common subset *(21)*. These data again underscore that, although ligand binding in two closely related receptors shares considerable similarities, it displays (on a very fine scale) many subtle differences that preclude direct extrapolation from one set of data to another.

4.2. Ligand Binding to the Receptor Extracellular Regions

Unlike small ligands (photon, biogenic amines, nucleosides, eicosanoids, lysophosphatidic acid, and sphingosine 1-phosphate), peptides bind to the extracellular domains of the receptor. Determinants have been identified in the receptor N-terminus that are essential for recognition of various peptides (90 amino acids), including oxytocin, vasopressin, endothelin, opioids, or substance P. Moreover, the long extracellular N-terminus of the target receptor constitutes the primary high-affinity binding site of large glycoprotein hormones (30 kDa) such as lutotropin/choriogonadotropin (LH/CG), thyrotropin-stimulating hormone (TSH) or follitropin-stimulating hormone (FSH). Upon ligand binding, this domain may undergo a conformational change that allows secondary contacts with extracellular loop regions and eventually leads to receptor activation (reviewed in refs. *6* and *22*). Note-

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Fig. 2 *(From opposite page)* squares. Residues forming the NPXXY motif on helix VII are indicated by white diamond symbols. Several other important residues for ligand binding and receptor activation mentioned in the text are also indicated in the figure.

(**B**) Organization of the transmembrane helices as seen from the extracellular side of the membrane. The helices are positioned according to the projection maps of rhodopsin and are believed to be organized sequentially in a counterclockwise manner. Conserved amino acids through class A receptors are indicated.

worthy peptides of rather small size (40 amino acids) show a mixed binding profile through additional transmembrane anchoring in addition to their primary interaction with the extracellular loops *(3,5,23)*.

Studies using point mutants and receptor chimeras clearly showed that the extracellular loops are also involved in receptor selectivity. Extracellular loop 2 appears to be a critical determinant to discriminate among α_1-AR subtypes *(24)*. In the case of μ-, κ-, and δ-opioid receptor types, extracellular loop 1 contains critical elements for μ-selectivity *(25)*, and extracellular loop 2 contains critical elements for κ-selectivity *(26)*. Extracellular loop 3, together with the external parts of helices VI and VII, is involved in δ-ligand selectivity by enhancing the affinity of the receptor for δ-ligands *(25,27–29)*. Additionally, this region also contains important determinants for μ-agonist selectivity *(30)* and for κ-selective alkaloids *(28)*.

4.3. Role of Extracellular Loop 2 and Conserved Disulfide Bridge in Small-Ligand Binding

Although binding of small ligands within the transmembrane core of the receptor is widely acknowledged, a possible involvement of the second extracellular loop has also been proposed for small ligands; however, this involvement is still under debate. Interestingly, two antagonists of the a_{1a}-AR, phentolamine and WB4101, exhibit unusual binding features in which three amino acid residues localized in extracellular loop 2 appear to be critical. This observation suggests a binding profile involving extracellular regions of the receptor that is more similar to what has been described for peptide hormone receptors *(24)*. In the high-resolution bovine rhodopsin structure, the second extracellular loop folds down into the binding-site crevice to form a lid over retinal *(12)*, and one may postulate a similar extracellular loop 2 structure exists in at least some class A receptors. Using the substituted-cysteine accessibility method (SCAM), Shi et al. *(31)* concluded that this may indeed be the case for the D2 dopamine receptor. Another argument in favor of a critical role for extracellular loop 2 comes from the observation that several antibodies directed to extracellular loop 2 induced AR and bradykinin receptor (BR) activation *(32)*. Nearly all class A receptors show the presence of two conserved cysteine residues that are believed to form a disulfide bridge connecting helix III and extracellular loop 2. However, the actual presence of the bridge has been established only in a limited number of cases, including rhodopsin *(12)*, μ-opioid *(33)*, leukotriene LTB4 *(34)*, muscarinic m1 *(35)*, platelet thromboxane *(36)*, TSH *(37)*, or gonadotropin-releasing hormone (GnRH) *(38)* receptors. This disulfide bond may be crucial for both the structural integrity and function of many GPCRs. Its

removal by mutagenesis severely disrupted ligand binding to muscarinic acetylcholine *(35)*, opioid *(33)*, and angiotensin (AT)1 *(39)* receptors and destabilized the high-affinity state of the β_2-AR *(40)*. The disulfide bridge likely dictates a relatively rigid architecture by constraining the extracellular loop. This, in turn, shapes the ligand-binding site, rather than contributing directly to ligand binding.

4.4. Relative Distribution of Agonist- and Antagonist-Binding Sites

Interestingly, antagonists are small molecules that invariably bind within the transmembrane region of class A GPCRs. They prevent agonist binding and subsequent receptor activation whether the agonist is a peptide or a small molecule. The generic antagonist binding pocket is located in a region flanked by helices III, V, VI, and VII, in which residues establish the main side-chain interactions with the ligand *(6,17,18)*. Receptor contacts with peptide agonists and nonpeptide antagonists do not substantially overlap at atomic levels in the tachykinin receptor NK1 *(18,41)*, AT1 *(3)*, or opioid receptors *(21)*. Therefore, competitive antagonism would primarily arise from a steric exclusion mechanism *(3,18)*.

5. RECEPTOR ACTIVATION UPON AGONIST BINDING

This section briefly reviews the models currently applied to describe GPCRs modus operandi. Attempts are made to draw a picture of the molecular events that occur upon agonist binding that lead to G protein activation. The role of palmitoylation is discussed. Finally, modulation of the interactions with intracellular partners is envisaged in the light of the receptor susceptibility to adopt multiconformational states.

5.1. Ternary Complex Models of Receptor Activation

In the original ternary complex model (TCM) described by De Lean et al. *(42)*, an agonist-bound activated receptor forms a complex with a G protein, resulting in its activation. This corresponds to a simple example of a receptor isomerization mechanism in which ligand-binding (A) promotes a conformation of receptor (R) that couples to and activates a G protein (G). The next level of progression toward present GPCR models involved the incorporation of different receptor conformations into the scheme. The demonstration of constitutive GPCR activity by Costa and Herz *(43)* indicated that receptors could couple to and activate G proteins in the absence of ligand. This required modification of the original TCM, which did not enable spontaneous formation of the R*G species; this modification resulted in the extended TCM (ETC) (ref. *44*; Fig. 3A). According to the ETC, the receptor

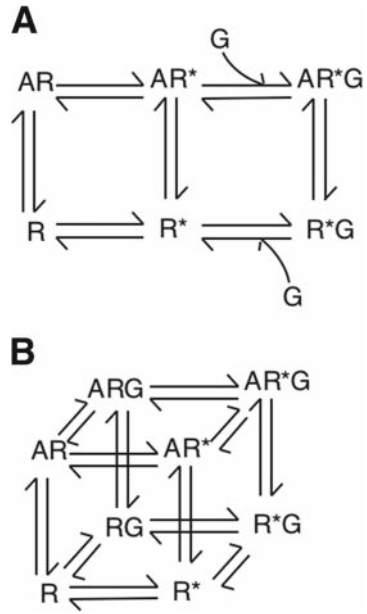

Fig. 3. Ternary models of G protein-coupled receptor (GPCR) activation. (**A**) Extended ternary complex model (ETC) proposed by Samama et al. (64). According to this model, the receptor can spontaneously adopt either an inactive (R) or an active (R*) conformation. Only the activated form (R*) of the receptor can interact with the G protein (G) in the presence or the absence (constitutive activity) of an agonist (A). (**B**) Cubic ternary complex model (CTC) proposed by Weiss et al. (65). In this more thermodynamically complete representation of GPCR activation, both the inactive state (R) and the active state (R*) of the receptor are allowed to interact with the G protein (G).

exists in an equilibrium between an inactive conformation (R) and an active conformation (R*). In absence of agonist, the inactive form R prevails, but a certain fraction of receptors spontaneously assume the R* state because of the low-energy barrier separating the two conformations. Agonists are predicted to bind with highest affinity to R* and to shift the equilibrium to a larger proportion of receptors under the active conformation. Conversely, inverse agonists that have the ability to inhibit agonist-independent activity (also called constitutive activity) stabilize the inactive conformation R, thereby shifting the equilibrium away from R*. On the other hand, neutral antagonists do not influence the equilibrium between R and R*.

In 1996, Weiss et al. *(45)* proposed a more thermodynamically complete model called the cubic TCM (CTC; Fig. 3B). In this model, both the active R* and the inactive R conformations of the receptor are allowed to interact with the G protein, whereas in the ETC model only the active R* receptor state could interact with the G protein. It is presently unclear which of these models better predicts and describes experimental findings with GPCRs. On the practical side, the ETC model has fewer parameters and is simpler to use, whereas the CTC model is more comprehensive but has a greater number of nonestimatable parameters. The choice for the appropriate model may be dictated by the importance of the inactive agonist–receptor–G protein (ARG) state: GPCR systems in which the ARG state is negligible can be accurately described by the ETC model, whereas other systems in which the ARG species plays a role (e.g., cannabinoid receptors *[46]*) require use of the CTC model (ref. *47*; Fig. 3).

Increasing evidence points to the existence of multiple conformational states for GPCRs (*see* Subheadings 5.3. and 5.4.). Additionally, experimental data indicate that neither the ETC nor the CTC model accurately describes the complex behavior of GPCRs. In an attempt to embrace the multiplicity of receptor conformations, multistate models in which the receptor spontaneously alternates between multiple active and inactive states have been proposed *(48,49)*.

5.2. What Do Constitutively Active Mutants and Rhodopsin-Based Models Tell Us About Activation Mechanisms in Class A Receptors?

Some mutations appear to enhance basal activities of GPCRs and, therefore, are believed to mimic the agonist activity and to favor the active state of the receptor. This, in turn, facilitates productive interaction with intracellular G proteins. These mutant receptors are currently called constitutively active mutants (CAMs). The δ-opioid receptor was the first GPCR described as able to modulate second messengers in the absence of agonist *(43)*. A fairly large number of CAMs were incidentally identified from mutagenesis studies on many different GPCRs. These CAMs contributed massively to the set of data that helps explain the mechanisms of receptor activation. The current hypothesis states that CAMs release the conformational constraints of the GPCR inactive state. This was first postulated for the α_{1B}-AR. Mutation of alanine 293 (A 6.34) and replacement by any of the 19 other amino acids generated a CAM, suggesting that the gain of function resulted from the loss of an intramolecular constraint *(50)*. Indeed, the current belief is that agonist binding to a wild-type receptor introduces new

molecular contacts that replace the intramolecular interactions constraining the receptor in an inactive conformation. This results in a conformational switch and subsequent receptor activation. However, many CAMs are likely activated by simple disruption of interactions that exist within the receptor inactive conformation, rather than by formation of new intramolecular bonds. Therefore, it should be remembered that the actual structure adopted by CAMs is only an approximation of the real active conformation of the receptor (for a review, *see* ref. *51*).

The crystal structure obtained for rhodopsin corresponds to the inactive form in which 11-*cis* retinal is bound, and this serves as a template to postulate movement of helices III, VI, and VII upon light activation. Class A GPCRs share a good number of conserved structural determinants with rhodopsin. Therefore, the high-resolution structure of rhodopsin has been used as template for GPCR modeling of the transmembrane domains, and the helix movement model has been extended to class A receptors as a common mechanism of activation. According to this hypothesis, ligands activate GPCRs by disrupting the networks of intramolecular contacts that stabilize the ground state. This modifies the conformation of the receptor so that it optimally exposes epitopes that bind and stabilize a conformation of the G protein close to the transition state for GDP–GTP exchange and G protein activation.

Despite the availability of a high-resolution structure of rhodopsin at 2.8 Å, the actual mechanism used to disrupt stabilizing intramolecular interactions remains elusive. Evidence for movements of helix VI relative to helix III have been essentially provided by several different approaches that were mostly applied to rhodopsin. Biophysical studies included Fourier transformed infrared resonance spectroscopy (FTIR), surface plasmon resonance (SPR), tryptophan ultraviolet (UV)-absorbance spectroscopy, and electron paramagnetic resonance spectroscopy (EPR) (reviewed in ref. *52*). Spectral changes were also measured upon *N*,*N*'-dimethyl-*N*(iodoacetyl)-*N*'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylene-diamine (IANBD) binding to cysteine residues in the β_2-AR *(52,53)*. Additionally, several indirect strategies were used, including generation of *bis*-histidine metal ion-binding sites between cytoplasmic extensions of helices III and VI in rhodopsin receptors *(54)*, β_2-ARs *(55)*, and NK1 receptors *(41)*. Cysteine accessibility was also determined in a β_2-AR CAM *(56)* and random mutagenesis was performed on muscarinic m5 *(57)*, δ-opioid *(58)*, AT_{1A} *(59)*, and C5A chemo-attractant *(60)* receptors.

In rhodopsin and biogenic amine receptors, one key event in the activation process may involve arginine (R3.50) in the highly conserved E/DRY

motif at the cytoplasmic side of helix III (Fig. 1). Protonation of this residue would disrupt the ionic interaction with a glutamic acid (E6.30) at position X_1 of a basic "X_1BB X_2 X_3B" motif (where B is a basic amino acid and X is a nonbasic amino acid) located at the junction region between intracelullar loop 3 and helix VI (Fig. 2A). Mutagenesis studies have established this mechanism for 5-HT_{2A} receptors *(61)*, H_2 histamine receptors *(62)*, α_{1B}-ARs *(63)*, and β_2-ARs *(64)*. Mutagenesis of residues clustered at the junction between helix 3 and intracellular loop 2 in the muscarinic m5 receptor suggested that some of the amino acids adjacent to the E/DRY motif are involved in maintaining the receptor in an inactive state but also alternate with residues required for G protein coupling *(65)*. A similar role in G protein activation was postulated for the N-terminus of intracellular loop 2 in rhodopsin *(66)* and more recently in the V_{1A} vasopressin receptor *(67)*. ^{1}H NMR analysis established a similar structure for the vasopressin and rhodopsin intracellular 2 loops *(67)* but was distinct from the α_{2A}-AR intracellular loop 2 conformation *(68)*. This is of particular interest, because unlike the other two, the α_{2A}-AR is not activated by mutation of the aspartate in the DRY motif and therefore diverges from the consensus model described earlier *(69)*.

In addition to the R3.50–E6.30 salt bridge, the residue X_3 (6.34) of the basic motif is hydrogen-bonded to the arginine R3.50 in rhodopsin *(12)*. Introduction of a lysine at position X_3 revealed that the residue at position 6.34 is also involved in constraining biogenic amine receptors in an inactive form in the α_{1B}-, α_{2A}-, β_1-, and β_2-ARs and in the 5-HT_{1B}-, 5-HT_{2A}-, and 5-HT_{2C}-receptors (refs. *61* and *70* and references therein). However, this strategy may not generalize across all receptors. In the case of opioid receptors, the ionic interaction postulated earlier between E6.30 (X_1 residue of the basic motif) and R3.50 (in the DRY motif) cannot occur, because the glutamate residue E6.30 on helix VI is replaced by a leucine. Moreover, mutation of T6.34 into a lysine does not activate the μ-opioid receptor *(70)*. These data show that the actual interactions depend on the residues and local environments at the intracellular ends of helices III, V, and VI and that sequence differences in this region are likely to support locally different forms of activation mechanisms *(71)*. Interestingly, in the δ-opioid receptor R258 (6.32), the second basic residue of the "X_1BB X_2 X_3B" motif would be involved in an ionic bridge with E323 (7.43) on helix VII *(58)*.

A group of mutations comprising tryptophan W173 (4.50) that is strictly conserved in all rhodopsin-like GPCRs (despite its location on the most variable helix IV) induced constitutive activation of the δ-opioid receptor *(58)*. This cluster of mutations could either directly or indirectly affect the orien-

tation of W173, which would play a central role at the helix II–helix IV interface in controlling the orientation and outward motion of helix III during the activation process. W173 is also involved in opioid ligand binding *(21)* and has been located within the binding crevice in the D2 dopamine receptor *(72)*. Because of its high conservation, W173 may represent a key switch for helix III movements in most GPCRs.

Chen et al. *(73)* reported that a phenylalanine F303 (6.44) on helix VI is a key residue involved in α_{1B}-AR transmembrane movement that leads to G protein activation. This residue is highly conserved among GPCRs and is located several residues below those identified as being important for ligand interaction and receptor activation in many GPCRs. A similar role has been assigned to the equivalent phenylalanine residue in chemo-attractant C5A *(60)*, muscarinic m5 *(57)*, and cholecystokinin receptors *(74)*. In the muscarinic m1 receptor, the conserved F374 (6.44) in helix VI is part of a network of interactions involving a leucine residue L116 (3.43) in helix III and the asparagine N414 (7.49) of the NPXXY motif on helix VII *(7, 75)*. Additionally, an important and specific interaction occurs in rhodopsin between the NPXXY motif and the methionine M257 (6.40) on helix VI *(76)*. In the δ-opioid receptor, mutation of the tyrosine Y318 (7.53) of the NPXXY motif into a histidine or replacement of methionine M262 (6.36) in helix VI by a threonine led to constitutive activity *(58)*. Interestingly, a residue equivalent to M262 is highly conserved among the peptide receptor family, and its mutation in the LH receptor is associated with precocious puberty in humans *(77)*. These data support the view that the conserved NPXXY motif plays a central role in the conformational switch that leads to receptor activation and underscore the importance of networks of hydrophobic interactions in maintaining GPCRs in the inactive state. Following agonist binding, these networks of Van der Waals interactions may be disrupted, resulting in the removal of the hydrophobic latch between helices III, VI, and VII. This, in turn, may induce a rotation of helices VI and VII relative to helix III. From the previous examples, it can also be concluded that although activation of class A GPCRs may be associated with similar conformational changes, different receptors may employ specialized sets of intramolecular interactions to produce these changes.

A whole-receptor random mutagenesis strategy applied to the δ-opioid receptor identified 30 mutations distributed throughout the receptor sequence and allowed researchers to draw a general picture of the events leading to receptor activation *(58)*. The N-terminus, extracellular loop 3, and upper portions of helices VI and VII constitute an outward platform that responds to extracellular ligands and initiates transmembrane signaling.

Movement of at least helices VI and VII throughout the transmembrane core then follows, in addition to local re-arrangement of the helices III, VI, and VII which are proposed for rhodopsin and several biogenic amine receptors. Again, a common structural switch might involve the cytoplasmic ends of helices III and VI identified in several class A receptors (histamine H_2 receptors, μ-opioid receptors, ARs, and muscarinic receptors).

Notably, this study identified five amino acid modifications in the N-terminal domain that enhanced spontaneous activity of the δ-opioid receptor (Q12L, D21G, P28L, A30D, and R41Q) *(58)*. Each mutation substantially modified the chemical nature of the amino acid side-chain, introducing or deleting ionic charges or modifying hydrophobicity and structural constraints. This suggests that the N-terminal portion of the receptor is folded as a domain whose structure and spatial orientation influences receptor function. This hypothesis is consistent with the rhodopsin structure, in which the N-terminal domain is folded as a β-sheet and covers the helical bundle like a lid *(12)*. Presently, functional activity of the N-terminal region has been investigated only in glycoprotein hormone GPCRs. For example, the N-terminal tail of the TSH receptor has been proposed to bind spontaneously to the empty receptor and act as an inverse agonist favoring the off-state *(78)*. The present data suggest that the short N-terminal domain of some class A GPCRs may also modulate the on–off transition.

5.3. Palmitoylation: A Modulator of Receptor Activity

Palmitoylation is a posttranslational modification that results in the attachment of a 16-carbon-long saturated acyl chain to a cysteine residue. Unlike other acyl chain additions, palmitoylation is a dynamic process. Several studies have suggested that dynamic palmitoylation could modulate receptor activity by influencing the coupling to G proteins as well as the receptor phosphorylation state.

Mutations of C-terminal cysteine residues have been reported for several GPCRs, and a variety of receptor functions were perturbed following these mutations *(79–81)*. These cysteine residues are often believed to be palmitoylated and, therefore, are involved in the formation of a fourth intracellular loop. Dynamic modulation of the local hydrophobicity through palmitoylation may uncover or mask receptor domains that govern interactions with intracellular effectors such as heterotrimeric G proteins or receptor kinases. For example, depalmitoylation of rhodopsin increased its ability to activate $G_t\alpha$-light-dependent GTPase activity *(82)*. Crystallographical data suggest that helix VIII serves in rhodopsin as a membrane-dependent conformational switch that may adopt a helical structure in the inactive state