BIASED SIGNALING IN PHYSIOLOGY, PHARMACOLOGY AND THERAPEUTICS

BIASED SIGNALING IN PHYSIOLOGY, PHARMACOLOGY AND THERAPEUTICS

Edited by

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Dedication

This book is dedicated to my wife Tiffany, my sons James and Alexander and my stepson Nicholas. You have been my inspiration, motivation and refuge; I am eternally grateful.

> . . .I will conclude with an hypothesis which – in its simplest message argues that biological communication consists of a complex meshwork of structures in which G proteins, surface receptors, the extracellular matrix, and the vast cytoskeletal network within cells are joined in a community of effort, for which my life and those of my colleagues is a metaphor.
>
> **Martin Rodbell 1994 Nobel Lecture**

Preface

As a beginning to this book, I would like to take a brief moment to explain the motivation and objectives of this text. The ultimate objective of the book is to provide a single but comprehensive source of information (for both personal and educational purposes) concerning signal transduction as it is understood in the present with the addition of a personal vision on receptor signaling.

The first chapter is purposefully basic and intended to provide both a general historical backdrop and description of basic pharmacological principles as a launching point to the more detailed descriptions of other topics provided by my co-authors. As the focus of the book is on common themes in receptor signaling across classes of receptors, there are natural redundancies apparent from chapter to chapter. With limited space, we have attempted to provide a comprehensive view of the field including understanding how we can model and assess signaling bias in the practical sense. I am motivated to put these topics together in order to provide a more complete view of how we envision receptor signaling and to put it in the context of evolution and a more universal view of receptor signaling. That is, despite clear and significant differences between classes of receptors, there are also universal principles to their function that have been selected and carried forward throughout evolution.

As this book is published, it occurs to me that the concepts of biased signaling are just coming to the popular forefront of the minds of the research world. However, in my mind, the contents of this book are a culmination of my thoughts and my journey in science.

There are several key moments in the development of these thoughts on signal transduction and the potential of multiple signaling outputs provided by activated receptors. The first being the observations made by my fellow graduate student, Tom Burris, in the late 1980s that dopamine could not only inhibit prolactin secretion from isolated anterior pituitary cells through Gi activation but also stimulate it through a Gs mechanism, and the discussions that followed within the lab that included Marc Freeman (my graduate advisor) and Béla Kanyicska. My exposure to research in nuclear receptors and the development of selective estrogen and progesterone receptor modulators at Wyeth also had a profound impact on my views since it was through this research that I came to the realization that cell background and associated proteins provided another level of regulation and possibilities for the development of better therapeutics. Lastly, the observation that naturally occurring ligands could also induce signaling bias in collaboration with Francisco López (on glycoprotein hormone receptors) and the development of synthetic biased agonists to the FSH and calcium-sensing receptors have rounded out this view.

In compiling this resource, I have sought out fellow authors who are well-respected experts in their domains and who have had significant impact on their research fields for the topics they provided. I would like to thank them for their commitment to this project and for the outstanding chapters they have written. It is my sincere hope that you, the reader, will find this book useful and compelling.

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About the Editor

Brian J. Arey received the Bachelor of Science degree in biology from Saint Louis University where as an undergraduate he performed research on estrogen and progesterone receptors and their interactions with chromatin. He then moved to Florida State University where he received both Master's and Doctorate degrees studying neuroendocrine physiology with Dr. Marc Freeman.

In 1992, his work in graduate school led to an National Institutes of Health (NIH) Postdoctoral Fellowship in the NIH Center for Reproductive Sciences at Northwestern University, where he studied the expression and signaling mechanisms of the mouse and rat prolactin receptors.

While at Wyeth in the mid-1990s, Dr. Arey collaborated to develop some of the first described allosteric agonists, antagonists and partial agonists to the follicle-stimulating hormone receptor (FSHR). As part of this research he was the first to demonstrate that the FSH receptor could activate multiple G-protein signaling pathways. In addition, he proposed the concepts of biased agonism/conformational dynamics as a natural physiological phenomenon in relation to glycoprotein hormone receptors.

Dr. Arey ultimately moved to Bristol-Myers Squibb where he and his colleagues developed allosteric modulators to a number of other receptors including novel allosteric modulators to the calcium-sensing receptor (CaSR). Throughout his tenure in pharmaceutical research, he has contributed to the discovery or development of multiple marketed medicines. Dr. Arey writes reviews and is requested to speak internationally on the topics of allosteric activation of GPCRs and the role of biased signaling.

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CHAPTER

1

An Historical Introduction to Biased Signaling

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OUTLINE

INTRODUCTION

Throughout human history, we have been intimately associated with our environment. As part of that environment, we contribute to the efficient ebb and flow of energy through the system. Similar to others within the environment, mankind has evolved and adapted to their surroundings, utilizing them as tools to prosper and continue the spread of the species. Just as we utilized stone for tools to cut wood or kill prey, so we also had an intimate knowledge and use of surrounding substances (most often plants) for medicinal purposes. Indeed, through millennia, the knowledge of medicinal substances was handed down and has grown. It wasn't until the modern era (ca 200 years) that we have harnessed

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the technical skill and understanding to develop synthetic medicinal substances. It is perhaps not a coincidence that the ability to develop synthetic medicines occurs in history simultaneously with the development of our understanding of physiology and pharmacology.

The concept of biological activity of endogenous and exogenous substances on the human condition is likely as old as the species itself. Study of these substances and the recording of them can be traced back as far as the Egyptian Empire where the oldest known record of pharmaceutical substances can be found within the Kahun Egyptian papyrus (Figure 1.1) that dates to ca 2000 B.C.[1] This text is found within a compendium of papyri dealing with many aspects of Egyptian life and is primarily a veterinary text, but also includes reference to gynecological issues, treatments, and midwifery. A more comprehensive text, the Ebers Medical papyrus dates to approximately 1550 B.C. and is a cumulative resource of medicinal treatments for many ailments.[1]

Despite the existence of various texts throughout the history of civilization, the knowledge of therapeutic substances was primarily passed down through oral history for millennia. Perhaps the first comprehensive modern text dealing with the use of medicinal substances which received legal distinction was the *Dispensatorium* from Valerius Cordi (ca 1546) that dealt with the specific synthesis of medicinal preparations for treatment of diseases and therefore represents the first modern pharmacopeia.[1]

For the majority of human existence the use of therapeutics to treat disease was focused on using natural remedies from plants and substances readily found within our environment. However, the modern era of synthetic therapies was realized through the slow development of technological and scientific advances that laid the groundwork for better

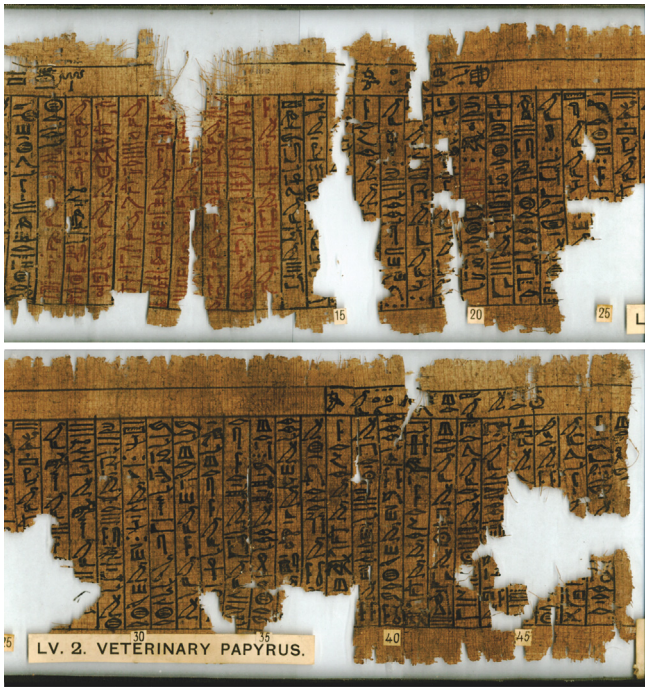

FIGURE 1.1 The Kahun Egyptian papyrus. The use of medicinal substances is probably as old as man himself. The earliest known written text discussing medicinal substances is found in the Kahun papyrus from Egypt. *Reproduced with permission of the Petrie Museum, University of London.*

understanding of biochemistry and cellular biology. It began with the realization of receptive substances within cells that could act as mediators of exogenous stimuli. The receptor theory was ushered in by two men, working independently but with a similar vision. The basis of their hypotheses was borne from a desire to understand how exogenous substances could impact cellular function. John Langley[2] and Paul Ehrlich[3] came to a similar conclusion but from different directions. Although Ehrlich is most often associated with the concept of receptive substances, it was Langley that perhaps had the clearer vision of the nature and utility of receptors.

Ehrlich was keenly interested in infectious disease and utilized some of the cutting edge techniques in the form of histology that the period had to offer. In the late nineteenth century, the germ theory was also gaining ground, and numerous reports had been published claiming that bacteria were able to produce anti-bacterial substances that would inhibit the growth of other bacterial species. In using the newly emerging histological techniques of the time, Ehrlich hypothesized that each cell contained a specific mixture of receptive substances that would bind the stains (e.g., methylene blue) with which he was working. He hypothesized that the bacteria that were taking up methylene blue contained "side chains" that interacted with the dye and allowed it to bind to the bacteria.

Langley was a neuroscientist interested in understanding the function of neurons and the neuromuscular junction. Initially he was interested in understanding the function of some of the paralytics available at the time. However, over time this research evolved into understanding the autonomic control of muscles and salivary gland secretions. Ultimately, this led to the study of the effects of various "poisons" on the neuromuscular junction.[4,5] His work was aided by other researchers of the time, including the previous work of Ehrlich and his theory of side chains, and the rapidly improving field of histology, especially the work of Ramón y Cajal that helped to elucidate the relationship of neurons to muscle fibers at the motor end-plate.[5] A key observation by one of his previous students, Thomas Elliot, helped to solidify his theory by demonstrating that application of exogenous substances (for example, extracts of the adrenal gland [adrenalin]) induced effects on the muscle similar to that elicited by electrical stimulation of sympathetic nerves. In his now famous publication, Langley not only hypothesized the existence of a receptive substance in muscle cells for adrenalin released by the sympathetic nerve, but also generalized this hypothesis to include the action of other compounds and other cell types:

> So we may suppose that in all cells two constituents at least are to be distinguished, a chief substance, which is concerned with the chief function of the cell as contraction and secretion, and receptive substances which are acted upon by chemical bodies and in certain cases by nervous stimulation. The receptive substance affects or is capable of affecting the metabolism of the chief substance.[2]

ISOLATION AND CHARACTERIZATION OF RECEPTORS

The study of pharmacology as a focus of research traces its origins to the work of Buchheim and Schmiedeberg during the mid to late nineteenth century and developed independently of the receptor theory being studied by Langley and Ehrlich. Bucheim and Schmiedeberg were focused on the understanding of the relationship between chemical

structure and biological activity, thus laying the foundation for study of different compounds and assessing their biological effects. For this reason, they are recognized as the fathers of pharmacology.[6] The study of pharmacology was a natural evolution from physiology, just as physiology evolved from the study of anatomy,[7] and it was Bucheim's vision of understanding how chemicals induced effects on tissues that would be extremely important to the development of improved therapeutics for clinical use.

Pharmacology can be defined in the modern world as "the science of drugs, their sources, appearance, chemistry, actions and uses".[8] This broad definition includes the understanding of synthesis, effects, structure−activity relationships, molecular interactions, metabolism and distribution, and therapeutic uses. It also reflects the evolution and expansion in biological knowledge of pharmacology that has occurred over the last 150 years (Table 1.1). We now realize the utility of understanding the principles of drug action on and off their intended target and this is due in large part to the early work of Bucheim and Schmiedeberg.

Bucheim and his student, Schmiedeberg, championed the study of chemicals on biological function against overwhelming doubt from the prevailing scientific community of the time. If Bucheim was responsible for the vision of the importance of understanding the action of chemicals on physiology, Schmiedeberg was responsible for executing this vision and for developing the experimental data to convince others that this new field of study was of immense value. In his 46 years at the University of Strasbourg, Schmiedeberg was incredibly prolific providing numerous important observations that led to the fundamental understanding of pharmacology, including his scientific approach to experimental design. Within the context of Bucheim and Schmiedeberg, and Ehrlich and Langley, it is easy to understand how isolated visions were coming together in the late nineteenth century to give rise not only to pharmacology but also the link between pharmacology and receptor theory. These visions led to a revolution in understanding how biological systems interact with the external environment and how this could be used to not only understand disease but also to treat it. This period of time represents not only one of the key points in human history for understanding physiology, but also for developing therapeutics to correct pathophysiology and ultimately improve the quality of human life. It set in motion a cascade of new discoveries that led to the development of new therapeutics and founded the basic principles that are used in drug discovery today.

Despite the fact that pharmacology allowed for the characterization and profiling of receptors using functional and binding assays, isolation of receptors as separate protein entities remained elusive for almost another 100 years. For this reason, the nature of membrane-bound receptors remained a hotly debated issue within the world of biochemistry and pharmacology. It wasn't until the early 1970s that the first receptor protein was finally purified and characterized by Jean-Pierre Changeux through his isolation of the nicotinic acetylcholine receptor in the electric eel.[9,10] Changeux was an important figure not only for this discovery but also because the roots of his science were in allosteric interactions of proteins. He played a key role in the field of enzymology in which he had hypothesized that enzymes could be acted upon by sites distal from their active sites thus introducing the fundamental principles of protein conformational dynamics.

Changeux hypothesized that enzymes *in situ* were fluid structures. In the absence of substrate, the three-dimensional structure of enzymes was not static but was changing within its microenvironment. Interaction of the enzyme with its substrate was able to

TABLE 1.1 Some Key Discoveries in the History of Pharmacology

Year	Investigator	Observation
1872	Oswald Schmiedeberg	"Father of modern pharmacology" created and led the first research institute devoted to pharmacology
1878	Paul Ehrlich	Proposed that there are specific chemical characteristics of cells that allow them to bind dyes
1878	John Langley	Suggested that pharmacological agents form complexes with cells
1897	Paul Ehrlich	Proposed that cells contain "side chains" for binding toxins
1905/1907	John Langley	Theorized the existence of "receptive substances" in cells and their utility for "chemotherapy" (i.e., therapeutics)
1933	Alfred Clark	Proposed the receptor occupancy theory
1948	Raymond Ahlquist	Suggested the existence of two adrenalin receptor subtypes (α and β)
1956	Robert Stephenson	Proposed the concept of "spare receptors" and efficacy
1964	Pierre Changeux	Developed the allosteric theory of protein regulation in enzymes
1970	Pierre Changeux	Isolated and characterized the nicotinic acetylcholine receptor and suggested an allosteric mechanism of toxins
1972	Martin Rodbell	Demonstrated the existence of a GTP-dependent transducer protein and proposed the theory of "signal transduction"
1980	Alfred Gilman	Isolated the first G protein and determined its regulation of adenylate cyclase
1983	James Black & Paul Leff	Proposed the two-state model of receptor activation dependent upon receptor conformation
1984	Herman Kuhn	Identified β-arrestin activity to modulate rhodopsin activation
1986	Robert Lekfowitz & Richard Dixon	Cloned and expressed the first receptor (β-adrenergic receptor), predicted the seven transmembrane structure of GPCRs
1995	Hinrich Gronemeyer	Solved the first crystal structure of a nuclear receptor ligand binding domain
2000	Krzysztof Palczewski & Masashi Miyano	Reported the first crystal structure of a GPCR (bovine rhodopsin)

stabilize the enzyme into its active conformation. Furthermore, he demonstrated that the product of the enzyme reaction had the ability to interact allosterically to stabilize inactive conformation(s) of the enzyme. These observations were important to his purification of the nicotinic acetylcholine receptor. Thus, his observations laid the foundation in many ways for our current understanding of receptor–ligand interactions and how we now view receptor signaling across all classes of receptors.

At the turn of the twentieth century, there was evolution of thought in all fields of science including chemistry and physics. As these disciplines made new advances, some physiologists pushed to apply some of their techniques, most notably mathematical modeling, to the

systems they studied.[7] Archibald Hill was a key member of this movement and worked to apply mathematical models to pharmacological data in order to better quantify the relationship between compound concentration and biological effect.[11] Indeed, his application of mathematics to model pharmacological data bears his name (the Hill equation) and can be found as an integral part of further refinement of modeling of pharmacology that took place in the following years. This includes the application of the mathematical principles of Hill that can be found within the modeling of enzyme kinetics that was published shortly thereafter by Michaelis and Menten[12,13] and in the equation developed by Langmuir to describe the saturable nature of gas adsorption by various substrates (e.g., metal).

The Hill equation is derived from the law of mass action at equilibrium for reversible chemical reactions first proposed by Guldberg and Waage, and later by van Hoft. This model simply states that for simple chemical reactions, the rate of forward and reverse reactions are not only dependent upon the concentration of reactants but also on their affinity, or chemical attraction, for each other. Applying this idea to pharmacology, Hill proposed that this equilibrium model would apply for chemicals and their association with proteins in or on a cell. He was particularly interested in understanding the effect of oxygen partial pressure on binding to hemoglobin. This model was applied by Langmuir to receptor–ligand interactions several years later in response to the development of the receptor theory of drug–receptor interactions by A.J. Clark.[7,14]

The Hill equation is shown in Figure 1.2. Reaction 1 represents the interaction between receptor, R, and ligand, L. For the sake of this equation, the concentration of R is considered constant, whereas the concentration of L can be variable but in excess to the concentration of R. This relationship implies that the concentration of R, and therefore the affinity of R for L, is rate limiting: Where $[L_nR]$ refers to the concentration of the ligand–receptor complex; $[R_0]$ is the total receptor concentration (receptor number); $[L]$ is the concentration of free ligand (for experimental purposes this is considered the concentration of ligand used); k_1 and k_2 are the rate constants of the forward (association between L and R) and the reverse (dissociation of L and R) reactions, respectively; K_d is the equilibrium dissociation constant of the receptor–ligand complex and is equal to k_1/k_2; n originally referred to the number of ligand binding sites on the receptor and is also referred to as the Hill coefficient or Hill slope factor; K_A is the concentration of ligand at which half of the receptors are occupied (if $n = 1$, it equals the K_d). K_A serves as a measure of affinity of the ligand for the receptor such that the smaller the K_A, the greater the affinity. The quotient, $[L]^n/([L]^n + (K_A)^n)$, is referred to as the fractional receptor occupancy.

Alfred Clark utilized the Hill equation in the development of his receptor occupancy theory. Specifically, he utilized the Hill equation to model his theory that the concentration

$$nL + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} L_nR \qquad \text{Rxn 1}$$

$$[L_nR] = [R_0] \cdot \frac{[L]^n}{[L]^n + K_d} = [R_0] \cdot \frac{[L]^n}{[L]^n + (K_A)^n} \qquad \text{Eq 1}$$

FIGURE 1.2 The Hill Equation. This equation applied the law of mass action of chemicals to the interaction between ligands and their receptors in or on cells.

of a ligand was proportional to the effect it produces. Most importantly, he hypothesized that a ligand's effect on a cell was due to an excess of ligand acting upon a limited number of receptors on/in the cell. Thus, by the Law of Mass Action, the concentration–response relationship should follow a simple hyperbolic function as had been shown previously for the adsorption of gases to a metal;[4] that is, in the presence of a limiting number of receptors, the system is saturable. This resulted in the first evaluation of concentration–response curves in context of the effect of a ligand on a living tissue. In his first description of this concept, Clark studied the ability of atropine to block the effect of acetylcholine on isolated cardiac ventricular muscle strips (Figure 1.3[15]). In this use of the Hill equation, $[L_nR]$ is replaced by effect of a specific concentration of a ligand (Yobserved) and $[R_0]$ is replaced by the maximal effect achievable by the ligand in the system being studied (Ymax). In Figure 1.3, Clark's data of the effect of atropine is reproduced from his original paper.[15] It is important to understand that Clark's theory did not model the existence of intracellular mediators (signal transducers) that could amplify the signal of receptor binding, since the existence of these mechanisms was not known at the time, and is therefore a simplistic model of receptor pharmacology, especially as it pertains to agonist responses. This led to frequent inconsistencies between the mathematical modeling of the interaction of ligand and receptor and the observed responses.

As a result of these inconsistencies, and with increasing knowledge of agonist–receptor signal transduction, more sophisticated mathematical models were developed that took into consideration a two-step model of efficacy: signal binding (formation of a physical receptor–ligand complex that serves as an activation stimulus) and signal transduction (an amplified intracellular response elicited by the newly formed complex). James Black developed the operational model of pharmacology based on this two-step process that is still used today to model *E/c* (agonist concentration, [A]) curves.[16] However, as we will see later, recent advances in understanding of ligand and receptor interactions as it pertains to signal transduction has led to newer and more detailed mathematical models to describe

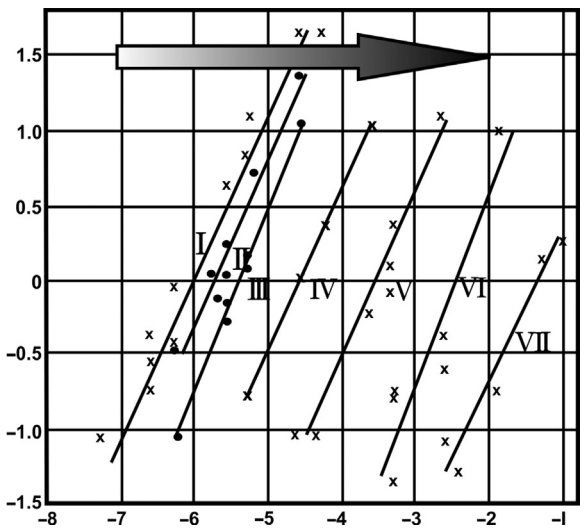

FIGURE 1.3 The effect of increasing concentrations of atropine to inhibit acetylcholine effects on isolated muscle (redrawn from[15]). Alfred Clark studied the effects of increasing concentrations of atropine on acetylcholine-induced muscular contraction in his landmark paper. He noted that as the concentration of the atropine increased (arrow) there was a rightward shift in the ability of acetylcholine to stimulate contraction.

these relationships to efficacy (see Chapter 3). These models are used today to accurately estimate the key characteristics of ligands on biological systems: potency and efficacy.

The understanding of receptors in the relationship of activation of biochemical changes within a cell was slow to develop following the development of the receptor and occupancy theories. Despite the demonstration of "receptive substances" and emerging pharmacological profiles of receptors to many ligands utilizing isolated tissues, the understanding of how the receptor–ligand complex actually induced an intracellular biochemical response was unknown. This was, in large part, due to the prevailing thought of the time that the cell was too complex a system to separate into workable pieces. This consensus prevailed until the 1950s when scientists began to develop ways to better understand the functional systems within cells. Again, development of new technologies and better techniques of isolating cells and cell components led to a new era in understanding of physiology and cell biology. This was a particularly productive time in cell biology and biochemistry, as noted by the many important discoveries of this time including metabolic enzyme pathways inside cells (e.g., Edwin Krebs) and the solution of the structure of DNA (Rosalind Franklin, James Watson and Francis Crick). It is important to note that until this period, and the research of Sutherland, the intracellular mechanisms altered by receptor binding to its ligand to elicit an effect were unknown.

Earl Sutherland was the first to isolate a signaling molecule, cyclic adenosine monophosphate (cAMP). Sutherland was interested in understanding the intracellular mechanism of epinephrine to stimulate glucose production in the liver. His initial work in this regard demonstrated that epinephrine stimulated activity of glycogen phosphorylase to produce glucose from glycogen. In a series of exquisite experiments, Sutherland and colleagues were able to isolate the membrane and cytosolic fractions of liver cells and showed that a heat stable compound was produced from epinephrine exposure to isolated liver membranes that, upon reintroduction to the cytosolic fraction, could induce activation of glycogen phosphorylase activity and increase glucose concentration. Working with his colleagues Ted Rall and Leon Heppel, Sutherland was able to isolate cAMP, show that it was the result of cyclization of ATP, and that addition of cAMP could mimic the effects of epinephrine treatment of liver cells on glucose production.[17,18]

Perhaps more importantly though, Sutherland realized the full magnitude of these observations and hypothesized the existence of "primary messengers" and "second messengers" (Figure 1.4). In Sutherland's view, the primary messenger is represented by the ligand binding to its receptor and the second messenger is represented by the activation or liberation of an intracellular mediator that was responsible for the observed biochemical and functional changes in the cell. These observations would ultimately give rise to the understanding and study of signal transduction. However, Sutherland's model of receptor signaling did not explain how the second messenger (in this case cAMP) was activated following receptor–ligand binding. In their view, Rall and Sutherland envisioned that each receptor not only served the purpose as a ligand binder but also as the liberator of the intracellular mediator; in this case cAMP.[19–21]

The term "signal transduction" was first coined by Martin Rodbell who was instrumental in uncovering the fundamental aspects of transferring the signal of ligand binding to its elicited biological response.[22] Rodbell utilized the concepts championed by Sutherland and adapted them based upon his unique view of cells. Rodbell viewed living cells and tissues as complex, natural computers that were comprised of discriminators, transducers,

and amplifiers. Receptors represented the discriminator and signaling molecules such as cAMP represented the amplifier (Figure 1.4). The existence of a transducer molecule that lay between the activation of adenylate cyclase and the bound receptor had been implied. The adipocyte with which Rodbell was studying responded to several different hormones and receptors that could induce production of cAMP. If each receptor was the liberator of the signaling molecule, cAMP, then the response to exposure of the adipocytes to multiple hormones simultaneously should be additive. Rodbell's group had shown it was not additive, suggesting that each receptor could activate a single pool of intracellular adenylate cyclase.[20,21,23] Through Rodbell's careful and elegant studies, he was able to identify the first transducer, which he termed G proteins due to their activity which required guanine triphosphate (GTP). In a series of seminal papers published in 1972, Rodbell demonstrated biochemically the existence of a transducer molecule that lay between the activated receptor and the stimulation of cAMP production by adenylate cyclase.

The discovery itself was serendipitous, in that Rodbell was studying the ability of ATP to uncouple the event of ligand binding to liberation of cAMP. In the process he also studied the ability of other naturally occurring nucleotides to do the same and found that GTP could uncouple signaling at concentrations three orders of magnitude less than ATP. He assumed correctly that the ability of his preparation of ATP to uncouple cAMP production must be due to contamination of the ATP with minute levels of GTP. Based upon his data, he postulated that the receptor–ligand complex stimulated activation of another membrane-associated protein that, in turn, activated adenylate cyclase to convert ATP into cAMP. The isolation of the first G protein from cell membranes was identified a few years later by Alfred Gilman who was strongly influenced by Rall and Sutherland.

Gilman and his colleagues utilized cultures of a mutant immortalized cell line, the S49 cells thought to be devoid of adenylate cyclase (so-called cyc(-) cells). In their experiments, they attempted to study the interactions between the adrenergic receptor and adenylate cyclase by reconstituting receptor-mediated activation of adenylate cyclase activity through combining membrane protein extracts between cyc(-) cells that possessed or didn't possess receptors. Through their careful design of complex and technically challenging

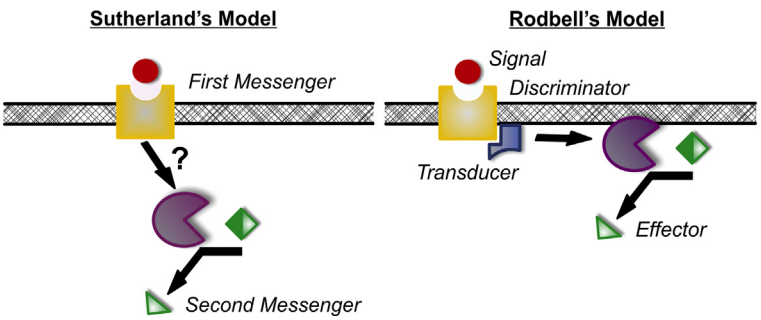

FIGURE 1.4 A comparison of Sutherland's and Rodbell's models of receptor-activated signal transduction. Sutherland's model isolated the first signaling molecule, cAMP, but did not understand that a transducing protein lay between the receptor and its production. Rodbell identified the existence of G proteins and envisioned receptor signaling similar to a computer where the ligand is the signal, the receptor is a discriminator, the G protein is the transducer, and cAMP is the effector.

experiments, Gilman and colleagues ultimately observed the existence of an additional membrane-bound protein that was required for receptor-activated adenylate cyclase activity and which required GTP.[24] Subsequently, they were able to also isolate and purify the subunits that constitute the G_s G protein. Interestingly, it was Rodbell who also hypothesized that receptors may have the ability to activate multiple signaling pathways simultaneously.[25–27]

The true structure of the G protein-coupled receptor itself would not be determined until 1986 when Richard Dixon and Robert Lefkowitz cloned and expressed a functional β_2-adrenergic receptor (epinephrine receptor, a GPCR), revealing its serpentine nature containing seven transmembrane domains, an extracellular amino-terminus, and intracellular carboxy-terminus.[28,29]

As noted earlier, the identity of signaling pathways elicited by both G protein-coupled receptors and ligand-activated ion channels occurred contemporaneously with initial important insights occurring in the late 1950s which gained momentum through the next decade until the elucidation of their signaling molecules in the 1970s. Interestingly, identification of the signaling molecules or initial steps following receptor activation preceded the identification of the receptor protein/gene itself.

MECHANISMS OF RECEPTOR FUNCTION

It is important to note that at the time of the elucidation of G proteins, Rodbell and his colleagues knew that the signal of receptor binding to ligand could be elicited by a single effector (cAMP). It is also important to note that these theories of receptor signal transduction applied to only what we refer to as G protein-coupled receptors. We now know that there are other receptor classes (Figure 1.5) and that each class has its own general mechanism for stimulating the production of effectors in order to alter cellular function/behavior. These include receptors that act as transcription factors (nuclear receptors), receptors that have endogenous tyrosine kinase activity (growth factor receptors), receptors that do not have endogenous enzymatic activity but stimulate tyrosine kinase phosphorylation cascades (cytokine receptors), and those that act as ion channels.

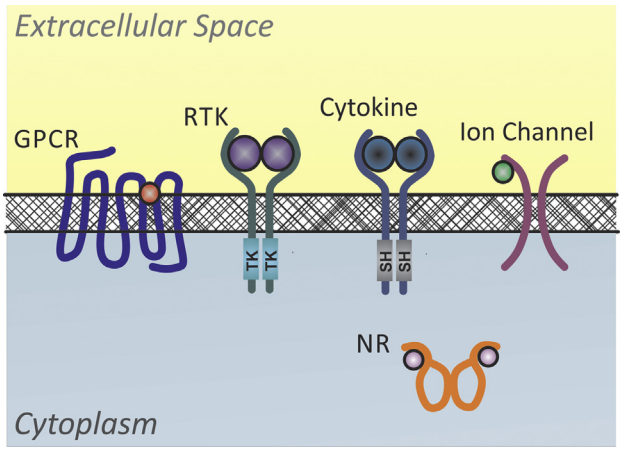

FIGURE 1.5 Receptor classes discussed. The general structure and cellular localization of the different classes of receptors discussed in this chapter are shown. Interaction of each receptor class with its particular ligand types is also shown.

GPCRs were originally thought to signal primarily through adenylate cyclase (cAMP) and phospholipase C (inositol triphosphate, IP_3), but with the identification of other G proteins it is well recognized that these receptors can also activate other signaling pathways. G proteins are heterotrimeric proteins comprised of a determinant α subunit and β and γ subunits that are shared with other Gα subunits (Figure 1.6). There are multiple isoforms of G_β and G_γ subunits but each isoform is not expressed in every cell; they have cell-specific gene expression patterns.[30] In addition to G_s (activation of adenylate cyclase), G_i (inhibition of adenylate cyclase) and G_q (activation of phospholipase C), there have been additional G proteins identified over the years including G_o, G_{11}, G_{12}, G_{13}, G_{15}, G_{16}, G_t, G_{olf}, and G_{gust}. Based upon their primary sequence homology and activities, they have been grouped into classes.[30] G_o primarily inhibits adenylate cyclase, similar to G_i, and along with G_i proteins makes up the $G_{i/o}$ class. G_{11} is similar in sequence and activity to G_q and therefore these proteins comprise the $G_{q/11}$ class of G proteins.

Table 1.2 provides a very general outline of the different G protein classes and their activities. In addition to inhibition of adenylate cyclase, some members of the $G_{i/o}$ class of G proteins also activate phosphodiesterases that act to metabolize cAMP. Another member of that class also can inhibit voltage-dependent cation channels, thereby regulating the polarity of the cell membrane. The $G_{12/13}$ class of G proteins acts to stimulate Rho and ERK phosphorylation cascades that are well recognized for modulating cellular growth and proliferation.

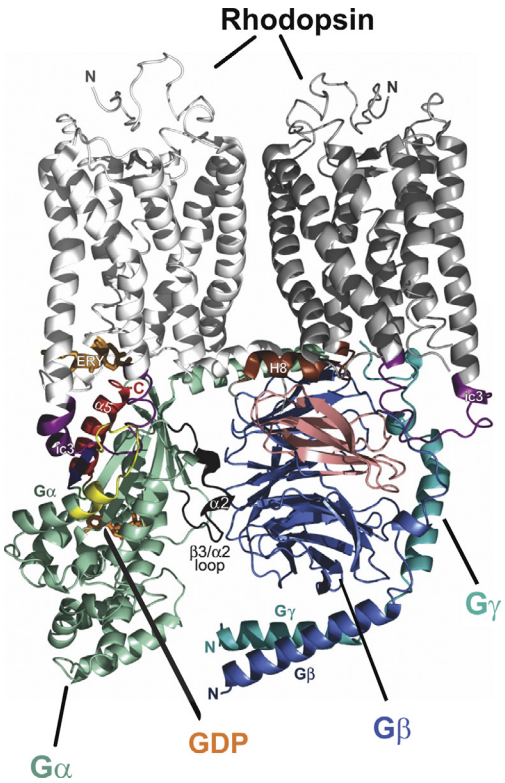

FIGURE 1.6 The crystal structure of a representative GPCR (rhodopsin) in association with G protein. The figure illustrates the areas of interaction between a dimer of rhodopsin and the various subunits of the G protein heterotrimer (α, β and γ) bound to guanine diphosphate (GDP). Note the interactions between intracellular loop 3 of one of the GPCR monomers (IC3) with alpha helix 5 of the G_α subunit and its proximity to the bound GDP. The IC3 of the other GPCR monomer makes contact with helices on the G_γ subunit.

TABLE 1.2 Activities of G Proteins

G Protein Class	# of Isoforms	Primary Signaling Effector(s)[30]
G_s	3	Activation of adenylate cyclase activity, liberates cAMP Calcium channel activation, increased intracellular calcium c-Src tyrosine kinase activation, protein phosphorylation
$G_{i/o}$	8	Inhibition of adenylate cyclase activity, decreases cAMP Activation of phosphodiesterases, decreases cAMP Inhibits voltage-dependent cation channels, c-Src activation
$G_{q/11}$	4	Activation of phospholipase C, liberates inositol phosphate RhoGEF activation, protein phosphorylation
$G_{12/13}$	2	Activation of Rho and ERK kinase pathways, protein phosphorylation
$G_{\tau}/_{g}$	2	Activation of phosphodiesterases
$G_{\beta\gamma}$	6β 14γ	Activation of β-catenin and ERK kinase pathways, receptor internalization

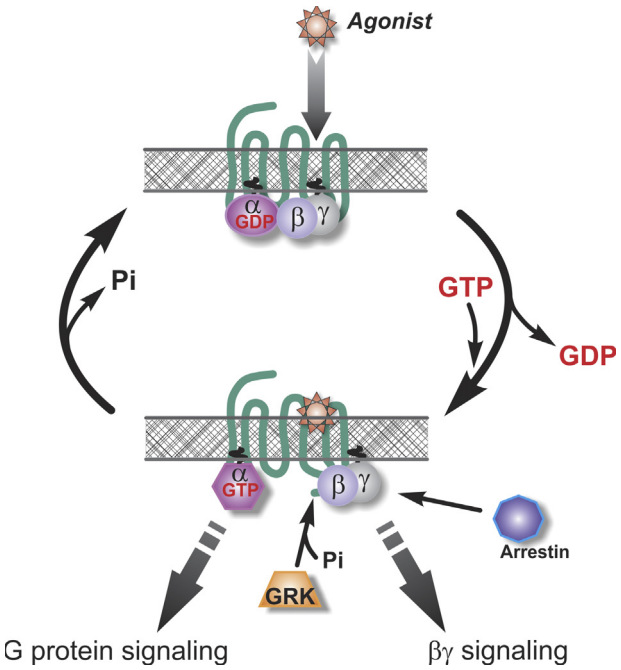

FIGURE 1.7 G protein activation by a GPCR in the presence of ligand. GPCRs act as GTP exchange factors. In the absence of ligand, the receptor binds the heterotrimer of G protein subunits (bound to GDP) through their intracellular loops; the interaction between the G protein subunits does not activate signaling since the βγ subunits act to inhibit exchange of GDP for GTP in the G_{α} subunit. When ligand interacts with the receptor, this causes conformational changes to be translated to the G protein heterotrimer that results in exchange of GTP for GDP on the α subunit, thus liberating active α subunit from the $G_{\beta\gamma}$ subunit. Each of the liberated subunits activates their own signaling cascades (Table 1.2). Activation of these cascades also activates G protein regulated kinases (GRK) that phosphorylate the GPCR, thus recruiting the desensitizing and signaling protein β-arrestin. Reassembly of the heterotrimer occurs following hydrolysis of GTP by G_{α} and is greatly enhanced by the presence of GTPase-accelerating proteins (GAPS) such as RGS (regulator of G protein signaling) proteins.

The steps to activation of G proteins upon receptor activation have been described in detail and numerous crystallographic determinations have been made of various G proteins in resting and activated states.[31] G proteins act as GTP exchange factors, whereby they associate with GPCRs as an intact heterotrimer comprised of one G_{α} subunit, one G_{β} subunit, and one G_{γ} subunit bound to guanine diphosphate (GDP)[19] (Figure 1.7). The signal of ligand activation of the receptor induces the exchange of G_{α}-bound GDP for GTP (Figure 1.7). This

exchange results in the breakdown of the heterotrimeric complex of G protein into two subunits: the free GTP-bound G_α subunit and the $G_{\beta\gamma}$ dimer. Since the protein−protein interaction between the β and γ subunits is so strong, and their proper protein folding that occurs during protein synthesis requires this interaction,[32] they are generally never found as monomers in the cytoplasm under physiological conditions.[31] Interaction of the GTP-G_α with effector enzymes associated with the local signaling complex in the cellular membrane requires hydrolysis of GTP to GDP. Once this occurs, the GDP-G_α subunit then recycles back to its original heterotrimeric complex with the $G_{\beta\gamma}$ dimer.

In addition to activation of signaling cascades by activated Gα subunits following GPCR ligand binding, the $G_{\beta\gamma}$ subunits also activate other signaling pathways. $G_{\beta\gamma}$ subunits can activate ion channels in the cell membrane, such as those for potassium, sodium, or calcium. In addition, activated $G_{\beta\gamma}$ subunits also can stimulate ERK kinase pathways. They can also activate pathways more commonly associated with G_q-stimulation, through activation of phospholipase and phosphoinositide-3-kinase enzymes.

Unlike GPCRs, growth factor receptors utilize their own innate tyrosine kinase activity to initiate the signaling cascade. These receptors generally bind large protein ligands. For this reason, they are also commonly referred to as receptor tyrosine kinases (RTKs). Both growth factor and cytokine receptors are comprised of an extracellular, ligand binding domain (LBD), a single membrane spanning domain, and an intracellular domain (Figure 1.8). In the case of growth factor receptors, the intracellular domain contains a protein sequence that is a tyrosine kinase, known as the kinase domain (TKD). The TKD contains numerous tyrosine amino acids that are critical to activation of the tyrosine kinase activity of the receptor. Growth factor receptors form dimers upon ligand binding. Dimerization of some growth factor receptors involves the ligand itself acting as a bridge between two receptor molecules. However, this is not the case for all receptors and some growth factor receptors dimerize following ligand binding, but the ligands are not directly involved in the protein−protein interactions stabilizing the receptor complex.

Some growth factor receptors are also known to oligomerize in the absence of ligand, suggesting that, at least for some receptors of this class, the ability to form complexes is an inherent property. This has also been found for other receptor classes including GPCRs. Receptor activation is initiated by ligand binding with a stoichiometry of 2:2; that is, two ligands stabilize a dimer of receptor molecules. The signal of ligand binding to RTKs induces activation of the tyrosine kinase domain of one of the receptors that subsequently phosphorylates the kinase domain of the other receptor[34] (Figure 1.9). Under resting conditions (no ligand present) the TKD of the receptor is under the influence of a cis auto-inhibitory mechanism that is induced by the intracellular juxtamembrane region of the receptor. Ligand interaction with the extracellular domain relieves this inhibitory influence allowing for a sequential auto-phosphorylation of key tyrosines within the TKD.

Furthermore, phosphorylation of the TKD of some of these receptors greatly increases the catalytic activity of that TKD, thereby enhancing their ability to phosphorylate the adjoining receptor TKD. The act of phosphorylation of the kinase domains of the receptor dimers then provides a newly formed site of assembly for the recruitment and subsequent activation of other signaling proteins. The recruited proteins have common structural motifs within them that recognize the phosphorylated receptors. Both Src homology domains (SH domain) and phospho-tyrosine-binding domains (PTB domain) recognize the phosphorylated tyrosine within the receptor intracellular region and associate with the receptor in a complex.[35,36]

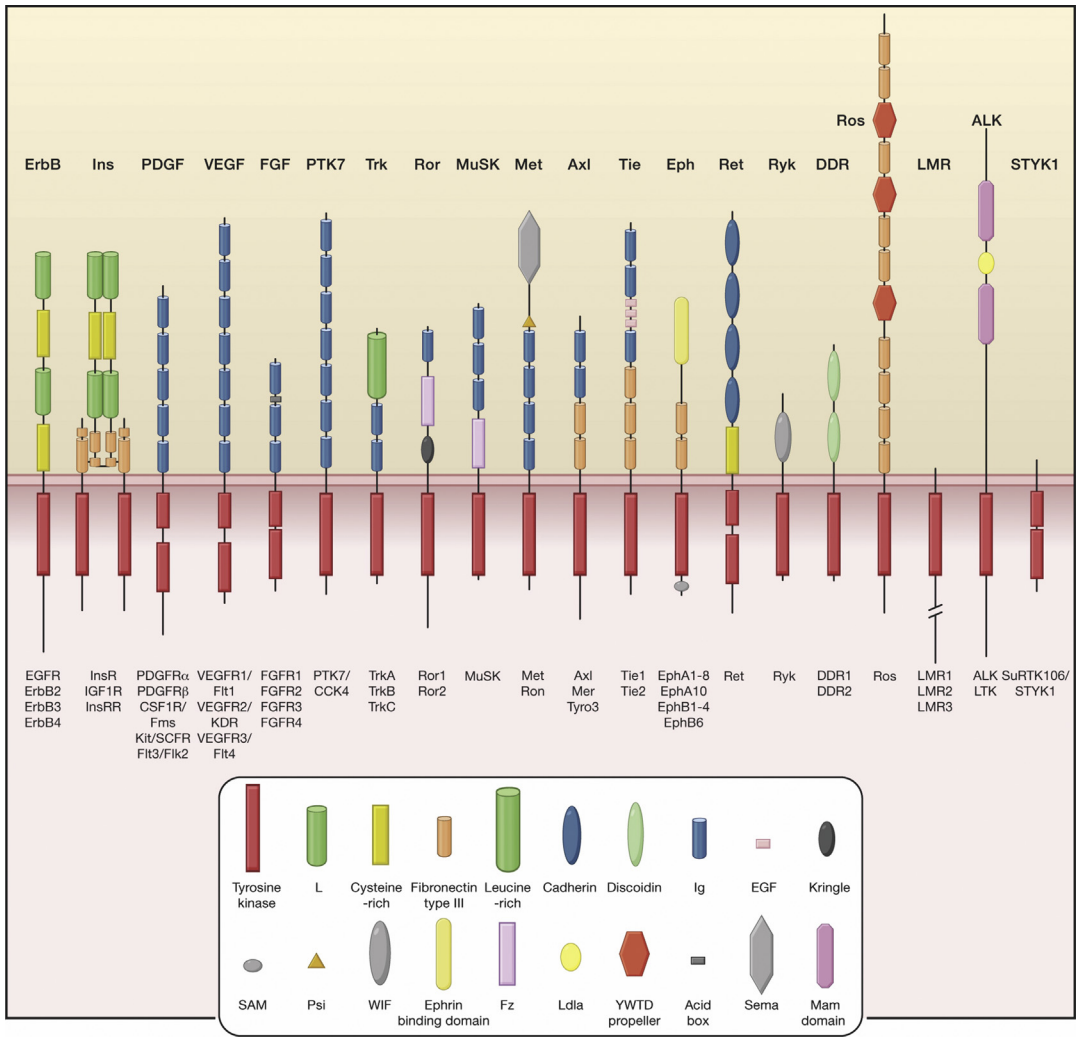

FIGURE 1.8 Comparative structures of the RTK family of receptors.[33] Overview of the general structural motifs present in the RTK family demonstrating the tremendous variability of structure exhibited by this class of receptor. The general structure of the monomers is shown, including the detail of the variable structural protein motifs found within the extracellular ligand binding domains. The various domains are shown as different shapes within each structure. In addition, the intracellular TKD of each receptor is highlighted. Republished with permission from Elsevier Limited, Oxford.

In this way, the ligand-bound, phosphorylated growth factor receptor behaves as a scaffold onto which is built a large macromolecular complex of signaling proteins (Figure 1.9).

Docking proteins associated with activated RTKs include FGF receptor substrate 2 (FRS2α), insulin receptor substrate 1 (IRS1), and the Grb2 (Growth factor receptor bound protein 2)-associated binder (Gab1). These docking proteins contain domains at their amino terminus that allow them to associate with the plasma membrane, thus allowing them to be

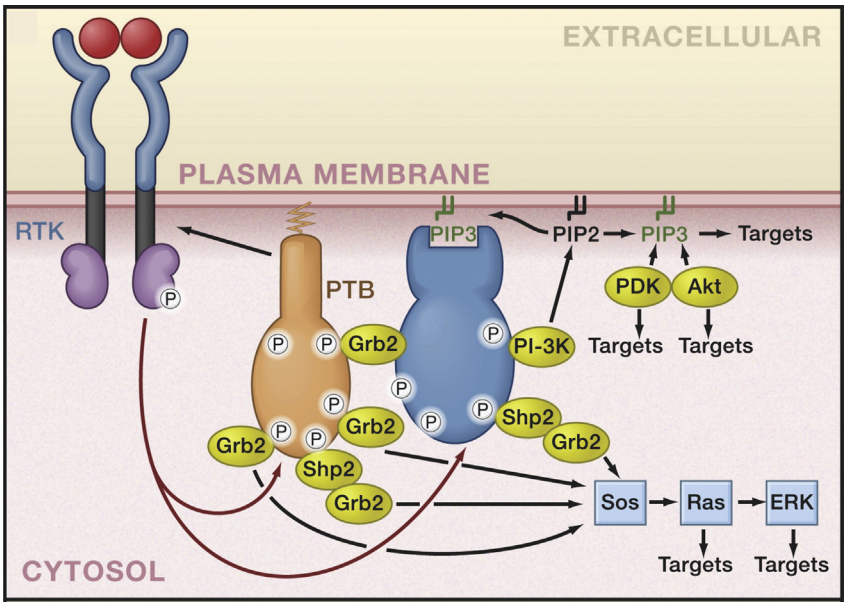

FIGURE 1.9 Signaling mechanism of RTK receptors.[33] The mechanism of ligand-dependent activation of RTK phosphorylation cascades. Ligand stabilization of a receptor dimer leads to activation of the tyrosine kinase activity of the receptor's intracellular TKD domain. Phosphorylation of closely associated docking proteins such as Grb2 and Shp2 acts to recruit other integral membrane protein signaling proteins, such as PTB and PI3K. Recruitment of PTB and PI3K leads to their activation via phosphorylation and this signal is translated by the PTB and PI3K proteins via their phosphorylation of signaling effectors within the cytoplasm (e.g., Sos, Ras and ERK). Reproduced with permission from Elsevier Limited, Oxford.

localized in close proximity to activate receptors (Figure 1.9). They also contain multiple tyrosine residues which are phosphorylated by the bound, activated RTK and are recognized by other signaling proteins such as Grb2, phosphoinositide-3 kinase (PI-3K), and Src homology phosphatase 2 (SHP2) (Figure 1.9). Phosphorylation of FRS1α induces association with both Grb2 and SHP2. These proteins then act to recruit another docking protein, Gab1 that, in turn, recruits additional signaling proteins (e.g., PI-3 K) to its surface via phosphorylated tyrosines. Other proteins can also be recruited.[33] Once bound to the docking site, these recruited proteins become activated to stimulate activity of other enzymes within the cytosol such as Sos, Ras, and ERK. These enzymes induce phosphorylation signaling cascades that regulate numerous biochemical activities of the cell including metabolism, protein synthesis, and gene expression. We can think of the activated RTKs as foci of signaling molecules recruited to interact via recognition of phosphorylated domains within their structures.

Unlike other receptor classes, the nuclear receptors (NRs) are not localized to cellular membranes, rather they are found intracellularly within the cytoplasm or nucleus. Due to their localization, they bind membrane diffusible ligands such as steroids, retinoids, fatty acids, and eicosanoids. Nuclear receptors have a general structure comprised of several domains that perform specific functions of the receptor (Figure 1.10 Schematic of general NR domains). The LBD and a DBD are the best conserved domains across the family of NRs. These two domains are connected by a flexible hinge region. The N-terminus and C-terminus

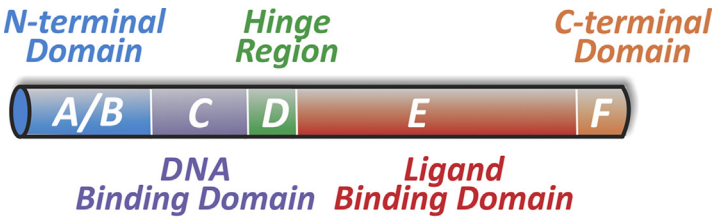

FIGURE 1.10 General structural domains of nuclear receptors. Nuclear receptors are comprised of a DNA binding region and ligand binding region that are connected by the hinge region. NRs generally function as dimers. These dimers, once formed in association with co-activators or co-repressors, bind to promoter regions on genes and regulate gene transcription. The dimers can be either homodimers (e.g., estrogen receptor [ER]) or heterodimers (e.g., RXR and retinoid activated receptor [RAR]). Depending upon the dimer formed, the interactions between the monomers are dependent upon the two partners and have a direct role in the effect of the receptors.

are variable across the family of NRs. As its name implies, the LBD is responsible for recognition and interaction with the ligand. The transcriptional activation and DBDs are responsible for binding and induction of gene promoters. The dimerization domain is the site of interaction between NRs. As can be inferred from their structural domains, NRs recognize membrane permeable ligands and dimerize. Upon activation they interact with specific sequences in the promoter regions of genes to induce or repress gene expression directly as part of an activating or repressing protein complex (Figure 1.10).

Similar to membrane-bound receptors, the NRs also utilize interactions with other proteins to fine-tune their functional activity. In this case, NRs interact in a specific fashion with additional proteins that help to assist them in forming the active transcriptional regulatory complex. These proteins, termed co-activators, recognize the ligand-bound receptor (Figure 1.11). Interactions between the co-activator and the NR induce specific structural changes, such that the complex specifically recognizes and binds to sequences of DNA in the promoters of the regulated genes. Once bound to DNA, co-activator-bound NRs recruit the transcriptional machinery required to drive production of mRNA by RNA polymerase. However, NRs can also repress gene transcription. In this case, the proteins associated with the NR are called co-repressors and in complex with the NR act to inhibit gene transcription once bound to their cognate promoter elements on DNA.

One key feature of co-activators and co-repressors is that they are expressed in a cell-specific fashion. Indeed, the particular repertoire of co-activators and co-repressors expressed in a given cell is specific to that cell type. Pharmacologically and therapeutically, this has been leveraged to produce synthetic ligands that act in a cell- or tissue-specific manner. Numerous synthetic ligands to NRs have been created that can induce tissue-specific gene regulation and in some cases these ligands can behave as agonists in one tissue and antagonists in another. Such tissue-selective receptor modulators are commonly prescribed drugs in some cases (e.g., tamoxifen, raloxifene, bazedoxifene). This has been utilized for the development of selective estrogen receptor modulators (SERMs) for hormone replacement therapies and for peroxisome proliferator-activated receptor (PPAR) therapies for insulin resistance and hyperlipidemia (e.g., rosiglitazone, fenofibrate). From a pharmacological perspective, this provides the ability to fine-tune drug responses in the context of multiple tissues, thereby producing wanted agonist activity in one tissue or cell type while producing antagonist or null activity in another.

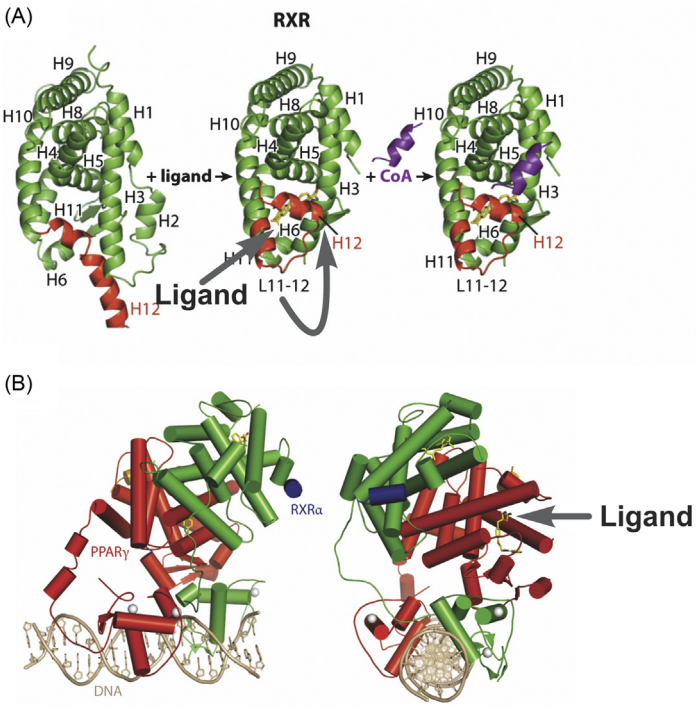

FIGURE 1.11 Crystal structures of an NR in complex with ligand and co-activator.[37] (A) illustrates the crystal structure of an NR (RXR) LBD in the absence of ligand, in the presence of ligand, and in the presence of ligand + co-activator. Note the movement of helix 12 (H12) and the subsequent juxtaposition of H12 and the ligand upon binding. This interaction then leads to the formation of a pocket for co-activator binding. (B) presents the crystal structure of an NR dimer (PPARγ and RXRα) bound to DNA. Note how the DBDs of the dimer wrap around and intercalate into the grooves of the DNA helix. *Adapted and reproduced with permission from Annual Reviews, Palo Alto, CA.*

Many of the mechanistic insights into how NRs work in the absence and presence of ligands and co-regulators have been gleaned from crystallographic analyses of isolated domains from the various NRs; however, Fraydoon Rastinejad and colleagues were able to develop crystals of an intact NR complex.[37,38] This crystal structure revealed key insights into how the NR heterodimer-co-regulator-DNA complex is arranged. They observed that the bound LBD in NRs has interaction with the DBD, thereby suggesting that not only can one achieve cellular/tissue specificity of NR activity via selecting specific co-regulator interaction but one can also actually drive DNA response element selectivity within a cell or tissue via the ligand−LBD conformation (Figure 1.11B). A similar structural relationship has been found for the retinoid X receptor and vitamin D receptor heterodimer in complex with DNA.[39] Therefore, NRs provide excellent opportunities to pharmacologically fine-tune the activity of the receptor with properly designed and directed synthetic ligands.

Ligand-gated ion channel receptors are also unique in their mode of action and signaling properties. Their main function is to modulate the balance of ions across the cell membrane. In the closed state ion channels are impermeable to the flow of ions. Each channel has its own specificity in terms of ion(s) that it is permeable to. Ion channels are found in all living cells and can be classified in several different ways. Generally speaking, ion channels are most often classified by their gating stimulus. Stimuli that induce permeability of channels to ions include membrane voltage (voltage-gated), exogenous ligands (ligand-gated), mechanical stretch (mechanosensitive), and even light (light-gated). For the purposes of this chapter we will focus on ligand-gated ion channels.