CELL SURFACE RECEPTORS

A Short Course on Theory and Methods

Third Edition

Lee E. Limbird

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by

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Research is essentially a dialogue with Nature. The important thing is not to wonder about Nature's answer–for she is always honest–but to closely examine your question to her.

A. Szent-Györgi, a paraphrase

PREFACE

In this, the third edition of *Cell Surface Receptors: A Short Course on Theory and Methods*, I have tried to link theoretical insights into drug-receptor interactions described in mathematical models with the experimental strategies to characterize the biological receptor of interest. I continue to need to express my indebtedness to my earlier tutelage in these areas by Pierre DeMeyts and Andre DeLean, which occurred during my postdoctoral years as a member of Robert J. Lefkowitz's laboratory at Duke University. Other concepts, particularly classical approaches to defining and characterizing receptors, I learned from Joel G. Hardman while teaching a course together at Vanderbilt School of Medicine on receptor theory and signal transduction mechanisms. My national colleagues also have been terrific teachers, including Terry Kenakin (Glaxo Smith Kline), Harvey Motulsky (GraphPad Software, Inc.) and Rick Neubig (University of Michigan). In the end, of course, the motivation of preparing such a text is for the students, whose contagious enthusiasm encourages efforts to meet their needs. I hope this text is of value to investigators–at whatever stage of their career they find themselves–who want to identify, characterize and understand the biology of a receptor of interest.

I prepared this revision just prior to taking a sabbatical from Vanderbilt University. Vanderbilt has been a wonderfully supportive and intellectually stimulating place to work and to continue to learn. I am grateful to Eric Woodiwiss, for his technical support in preparing the manuscript and the figures, and to Harold Olivey, Ph.D., a former student in my courses at Vanderbilt, who read and thoughtfully critiqued the text. Without their help, I suspect this edition would not have materialized from draft to completion.

The study of receptors has changed considerably over the period of the publication of the three editions of this book. The cloning of several genomes makes it unlikely that preparations of receptors now or in the future will arise from their purification as trace proteins from native tissues, but rather from a myriad of molecular approaches. Nonetheless, understanding the molecular mechanisms and ultimately the *in vivo* biology of these receptors means that investigators will engage in molecular, cellular and ultimate in vivo strategies. To work across this continuum means that we must be forever grateful to the remarkable insights of those early describers of receptor theory and the criteria expected for biologically relevant receptors. We are the beneficiaries of their genius, simply fleshing out a skeleton, a conceptual framework, that preceded us by decades.

Lee E. Limbird
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A good question is never answered. It is not a bolt to be tightened into place but a seed to be planted and bear more seed toward the hope of greening the landscape of idea. ***John Ciardi***

CONTENTS

1. INTRODUCTION TO RECEPTOR THEORY

Much of the conceptual framework regarding how to study receptor function evolved from pharmacological investigation of drug action. Consequently, the historical account of the development of receptor theory in this chapter will emphasize early investigations of drug action rather than (for example) physiological studies of hormone action. However, the reader must keep in mind that the term drug can be defined as any chemical agent that affects living processes. Drugs bind to receptors presumably designed for interaction with endogenous hormones and neurotransmitters or other regulatory agents. **Agonist** drugs are analogous to endogenous hormones and neurotransmitters in the sense that they elicit a biological effect, although the effect elicited may be stimulatory or inhibitory. Different agonists activate receptors along a continuum of effectiveness; those which induce or stabilize less productive conformations are termed **partial agonists**, a property which will be discussed in considerable detail later in this chapter. In contrast, **antagonist** drugs are defined as agents that block receptor-mediated effects elicited by hormones, neurotransmitters, or agonist drugs by competing for receptor occupancy. Antagonists, as initially defined, were competitive inhibitors of receptor occupancy by agonists, having no intrinsic activity in their own right. However, more recently, antagonist agents have been observed to have negative intrinsic activity, or behave as **inverse agonists**, and decrease

"basal" (agonist-independent, or constitutive) receptor activity. Still other antagonists of function mediate their effects by interacting with another, *allosteric*, site rather than in the binding pocket of the native agonist (defined as the *orthosteric* site) (Christopoulos and Kenakin [2002]; Kenakin [2004]; Neubig et al. [2003]). The properties of agents that interact via the orthosteric binding sites of the receptors are shown schematically in figure 1-1.

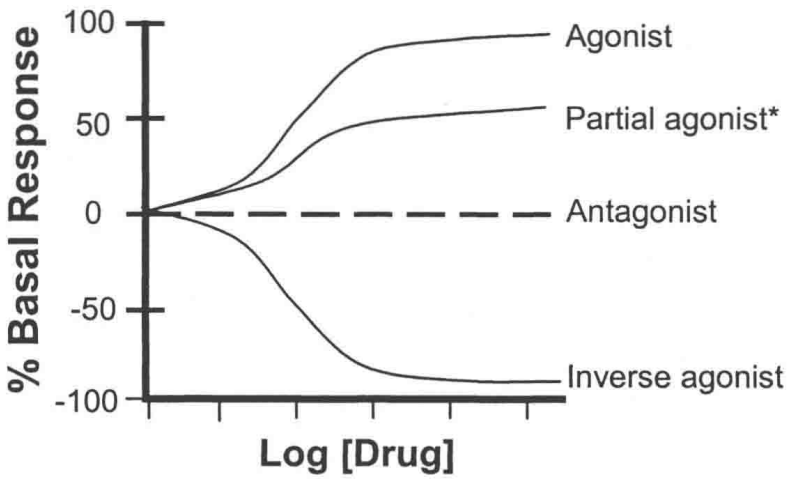

Figure 1-1. **Schematic representation of the functional consequences of the binding of drugs at the site of binding of endogenous ligands (the orthosteric site).** Agents which activate the receptor are *agonists*, and can elicit fully efficacious or partially efficacious (*partial agonist*) properties. *Partial agonists can either elicit a full response, but with lower efficiency or efficacy than full agonists, or, as shown in this schematic, elicit a submaximal response compared to a full agonist, even when fully occupying the receptor population. The properties of partial agonists and the theories that describe their behavior are considered in detail in later sections of the chapter. Classic, or null, *antagonists* occupy the agonist binding pocket and block receptor-mediated function by blocking agonist occupancy and subsequent agonist-elicited responses. *Inverse agonists*, or negative antagonists, stabilize inactive receptor conformations and decrease "basal" receptor activation in a dose-dependent manner.

ORIGIN OF THE RECEPTOR CONCEPT

Contemporary scientists take it as a "given" that biological substances such as hormones and drugs elicit their effects via interaction with specific *receptors* in a manner analogous to the interaction of substrates with enzymes. This

dogma was not always self-evident, but evolved from the remarkable insights of early scientists exploring a number of fundamental living processes.

Although Claude Bernard (1813-1878) never used the term *receptor*, he pioneered a pattern of scientific investigation that permitted clarification of the specificity and selectivity of drug action, particularly in regard to the *locus* of a drug effect. Bernard had a very unpretentious question: he simply wanted to know how the arrow poison curare worked. It was effective when "administered" by an arrow but, interestingly (at least to Bernard), was ineffective when taken by mouth. His early studies explained the importance of the route of administration of this drug for its lethal effects by demonstrating that although curare was unaltered functionally by saliva, gastric juice, bile, or pancreatic juice, it was not absorbed by the gastrointestinal tract, thus accounting for its harmlessness when swallowed. Bernard then wanted to understand just how curare effected its lethal paralysis. It was his impression from general observations that curare did not affect the sensory nerves, but instead altered motor nerve function. By an ingenious group of experiments, he determined that curare blocked the ability of motor nerves to control muscular contraction. Bernard noticed that, after injecting curare under the skin on the back of the frog, the frog showed progressively fewer reflex movements. If he skinned the hind legs of the frog that had been exposed to curare and isolated the lumbar nerve, he could produce no contraction of the leg muscles by stimulating the nerve electrically, whereas he could produce violent contractions if the same electrical stimulus were applied directly to the muscle. Bernard concluded from these experiments that muscle contractility is distinct from the nervous system that produces it and that curare removes the neural control of muscular function (cf. Bernard [1856]).

Bernard did not talk about receptors *per se*, but he did demonstrate that the ability of a drug to elicit its effects depends on its access to a particular location. As a result of his findings, Bernard encouraged investigators not to focus studies of drugs on organs but on organ *systems*, for example, the nervous system or the muscular system. Similarly, he believed that the mechanism of drug toxicity would be better elucidated by focusing on the drug-mediated death of these organ systems, rather than on the death of the organ itself. His own experiments revealed the existence of a neuromuscular "junction" prior to the demonstration of the muscular endplate as a discrete anatomic structure.

It may have been a physicist, rather than a physician or biological scientist, who first provided evidence for *molecular interactions* between two substances that had physiological consequences; Stokes (1864) observed that spectral changes occurred when oxygen was removed from, or subsequently reintroduced to, blood, implicating a complex between oxygen and hemoglobin. However, the biological concept of receptors is generally

attributed to Paul Erhlich (1854-1915), although the word **receptor** (receptive substance) was coined by one of Erhlich's contemporaries, J. N. Langley. Erhlich was a remarkable individual whose scientific career spanned (and even spawned) several biomedical disciplines. One overriding principle was common to all of Erhlich's investigative endeavors, and that was **selectivity.**

Erhlich's earliest work involved the distribution of lead in the body, particularly its preferential accumulation in the central nervous system. He had been inspired by a publication of Heubel on lead poisoning, which demonstrated that there were significant differences in the amount of lead found in various organs of animals that had succumbed to lead poisoning. When Heubel exposed the isolated organs of normal animals to dilute solutions of lead, the organs demonstrated the same differential uptake of lead as had been noted *in vivo*. In Erhlich's continuation of these studies, he realized that it was impossible to use a microscope to determine the basis for this differential selectivity of lead uptake in different tissues. Consequently, he changed his experiments to investigate the differential staining of tissues by dyes, as this could be easily detected. He continued to pursue the question of the basis for selectivity, from a more general standpoint. Erhlich's studies on dye distribution originated the concept of "vital staining," and his morphological distinction of leukocytes as acidophilic, basophilic, neutrophilic, or non-granular (based on the relative uptake of dyes of varying chemical constitution) is still in practice today. It was Erhlich's impression that although staining of dead tissue gave information regarding its anatomical structure, the staining of live tissue (i.e., "vital staining") provided insight into the properties and functions of living cells.

Erhlich's most acclaimed studies were his subsequent experiments in immunochemistry, cited as the basis for the Nobel Prize in Medicine awarded to him in 1908. By neutralizing the activity of toxins following incubation of toxins with anti-toxins in a test tube, Erhlich demonstrated that antigen-antibody interactions are direct chemical encounters and not generalized phenomena requiring the biological processes ongoing in a whole animal. From these observations Erhlich developed his "side chain theory" to explain the chemical basis for the immune response. He described the antigen as possessing two active areas: the haptophore (which functioned as the anchorer) and the toxophile (which functioned as the poisoner). He postulated that mammalian cells possess "side chains" that are complementary to certain chemical groups on the haptophore domain of the antigen, and thus serve as the basis for "anchoring" the antigen to the cell. This side chain-haptophore interaction thus gives the "toxophile" portion of the antigen access to cells that possess the appropriate side chains. Pictures reproduced from Erhlich's original notebooks show the side chains drawn with $-NH_2$ and -SH moieties, thus underscoring his assertion that the basis for these selective interactions between antigen and antibody was a chemical one. Quite clearly, his side

chain theory also could explain earlier observations concerning the preferential uptake of lead into the central nervous system and the principle governing vital staining of living cells. Erhlich conjectured that the normal function of cellular side chains was the binding of cell nutrients, and that the affinity of toxic substances for these groups was the fortuitous analogy between the structure of the exogenous toxic substance and the endogenous nutrient. Inherent in Erhlich's side chain theory was the burgeoning concept of specific cell surface receptors as the basis for targeting bioactive agents to the appropriate cell for response.

Erhlich turned his attention from large molecules, such as toxins, to low molecular weight molecules in a series of investigations that earned him recognition as the "father of chemotherapy" (see Albert [1979]). He believed that since the pharmaceutical industry could produce a number of small molecules (e.g., analgesics, antipyretics, and anesthetics) which appeared, at least functionally, to differentiate among various tissues in human beings, it also should be possible to design small molecules that differentiated between human beings and parasites (Erhlich [1913]). His initial studies pursuing this postulate shifted from the protozoan (*Trypanosoma*) to the bacterium (*Treponema*) when Hata showed that the latter organism could produce syphilis in rabbits. Thus, with a model system allowing more detailed studies of chemotherapeutic principles, Erhlich invited Hata to leave Tokyo and join him as a colleague in Frankfurt. Erhlich realized that a particular organism (i.e., *Trypanosoma* versus *Treponema*) was not critical for furthering his studies, because the basis of his experiments on differentiating host from parasite relied only on a *general* principle: that the parasite, as an incessantly motile organism, had a higher rate of metabolism than its host and presumably would be differentially sensitive to the toxic effects of arsenicals. Erhlich's work with a family of arsenical compounds revealed that agents were never entirely specific for the parasite (i.e., he never found his "magic bullet") and, at increasing concentrations, all agents studied had deleterious effects on the host. As a result of this finding, he introduced the term **chemotherapeutic index**, which he defined as the ratio of the minimal curative dose to the maximal tolerated dose. Second, Erhlich maintained that the haptophoric and toxophilic principles that guided immunochemistry also pertained in chemotherapy. Thus, he believed that small molecules also possessed distinct domains for binding to the target cell versus taking part in cellular nutrition or respiration. His own studies established that the arsenoxide group of arsenicals was essential for the lethal effect of these agents and that the chemical substituents on the arsenoxide group were responsible for uptake of the agent. The need first to "bind" the arsenical explained the basis for resistance to arsenicals by particular strains of trypanosomes, i.e., these strains were unable to recognize certain substituents on the phenyl ring attached to the arsenic.

All of Erhlich's studies on the basis of selectivity often are distilled into his often-quoted dictum, *corpora non agunt nisi fixata* (agents cannot act unless they are bound). Consequently, Erhlich's own advice regarding the pursuit of chemotherapeutic agents was to focus on the haptophore group, as it was the *conditio sine qua non* for therapeutic action.

J. N. Langley (1852-1926), of Cambridge University, was a contemporary of Erhlich who studied the chemical basis for autonomic transmission and neuromuscular communication. Langley extended Bernard's studies, which identified curare as a blocker of neuromuscular transmission, by demonstrating that curare also blocked chemical stimulation of the frog gastrocnemius muscle by nicotine, even after severance and degeneration of its motor nerves. However, even under curare "blockade" direct electrical stimulation of denervated muscle could elicit contraction. The mutually antagonistic effects of curare and nicotine, as well as the ability of direct electrical stimulation of the muscle to bypass the effects of curare, led Langley to conclude that nicotine and curare act on the same substance, which is neither nerve nor muscle. Langley called this postulated substance the "receptive substance" (Langley [1909]). The concept of mutual antagonism implying a common site of action was noted by Langley as well as by other contemporaries (e.g., Luchsinger in 1877 and after) for the effect of pilocarpine (agonist) and atropine (antagonist) on contraction of the heart (1909) and on secretion of saliva from the submaxillary gland of the dog (1878). Luchsinger was the first to apply the term "mutual antagonism" to the observed counter-regulatory effects. (See Langley [1878] for a translation from the German of Luchsinger's results and interpretations.) However, Langley emphasized that mutual antagonism depended on the relative concentrations of drugs added and that it had limits. For example, he observed that if he applied extremely large doses of pilocarpine to the artery of the submaxillary gland, secretion was blocked, i.e., pilocarpine could be made to mimic the physiological effect of atropine. Langley also realized that limits to mutual antagonism might be dictated not only by the properties of the receptive substance but also by other secondary effects of the drugs, such as drug-elicited changes in blood flow.

In summarizing his experimental findings, Langley concluded that the effects of the drugs he had observed could reasonably be assumed to result from the existence of some substance(s) in the nerve endings or glands with which both atropine and pilocarpine are capable of forming "compounds." He further postulated that these compounds (complexes) are formed according to some law by which the relative concentration of the drugs and their affinity for the receptive substance are critical factors. Thus, Langley first stated the concept of drug-receptor interaction and predated the algebraic description of these interactions as a consequence of mass action law. Langley observed that the height of the contraction elicited as a result of nicotine interacting with a

receptive substance depends on the *rate* of combination of nicotine with this substance as well as the duration of the resulting contraction, and that "saturable" effects on contractility could be observed. Langley actually postulated that if the combination of nicotine with the receptive substance were slow enough and the duration of contraction brief enough, a complete saturation of the receptive substance might occur without eliciting a visible contraction (Langley [1909]).

Despite persuasive evidence that receptors that are specific for particular drugs or endogenous substances do exist and thus determine the selectivity of biological responses to these agents, not all contemporaries or successors of Erhlich and Langley concurred. H. H. Dale (1875-1968) believed that the differential effectiveness of adrenaline analogs in mimicking sympathetic functions in varying tissues could be due to a chemical process, and did not necessarily imply the existence of specific chemical receptors on target tissues. He stated in 1910 that it was equally probable that the limiting factor determining the selective response to various substances might be the ease with which those substances reached their site of action. Thus, he appeared to favor the distributive rather than the interactive properties of a drug in determining its target cell selectivity, although Dale himself acknowledged that his own results could provide no decisive evidence one way or the other (cf. Dale [1914]).

OCCUPANCY THEORY

A. J. Clark (1885-1941) introduced a more quantitative approach to the description of receptor selectivity and saturability (Clark [1926a,b]). Based on his studies of antagonism between acetylcholine and atropine in a variety of muscle preparations, Clark postulated that drugs combine with their receptors at a rate dependent on the concentration of drug and receptor, and that the resulting drug-receptor complex breaks down at a rate proportional to the number of complexes formed (Clark [1927]). This statement implied that drug-receptor interactions obey the principles of mass action and thus could be described by the same isotherms used by Langmuir to describe adsorption of gases onto metal surfaces. Based on Clark's principles, a mathematical expression can be provided to describe drug-receptor interactions:

$$\text{rate of combination} = k_1 A\,(1 - Y) \tag{1.1}$$

$$\text{rate of dissociation} = k_2\, Y \tag{1.2}$$

where k_1 = rate *constant* for combination
k_2 = rate *constant* for dissociation