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Robert J. Lefkowitz

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A Magnificent Time With the "Magnificent Seven" Transmembrane Spanning Receptors

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The largest, most ubiquitous, and most versatile of the receptor gene families is that which encodes the seven transmembrane spanning receptors. In the cardiovascular system, such receptors regulate, for example, the rate and force of cardiac contraction, peripheral arterial resistance, and various aspects of renal function. The larger superfamily of receptors also regulates everything from sensory perception (vision, smell, taste) to hormonal and neurotransmitter signaling, to immune functions such as chemotaxis. There is virtually no area of human physiology in which they are not implicated. A majority of prescription drugs sold target such receptors either directly or indirectly. In the field of cardiovascular medicine, this is exemplified by α- and β-adrenergic receptor agonists and antagonists, angiotensin receptor blockers, and angiotensin-converting enzyme inhibitors.

A profound alteration in our understanding of these receptors occurred during the 1970s and 1980s, which transformed everything from the way they are viewed, to how they are studied, to how new drugs are discovered. I provide here a very personal recollection and perspective of my own voyage of discovery during this time, of its scientific antecedents, and of the technical and conceptual barriers that needed to be scaled.

The notion that biologically active substances initiate their actions by binding with high affinity and selectivity to some "receptive substance" on cells dates back about a hundred years. Early work of Ehrlich on interactions of antigens with cells and of chemotherapeutic agents with their targets was made even more explicit by J.N. Langley and H.H. Dale who, during the earliest years of the 20th century, studied actions of cholinergic and adrenergic agonists and antagonists on various target organs. Based on this early work, the period from about 1920 to 1970 was characterized by the development of classical theories of receptor action based on the law of mass action. This work, carried out with intact physiological preparations, was done by such giants of pharmacology as Clark, Ariens, Stephenson, Black, and Furchgott.

The 1960s and 1970s witnessed the beginning of a merger between receptor pharmacology and biochemistry, led by individuals such as Earl Sutherland. His demonstration that a proximate consequence of agonist activation of receptors, such as those for adrenaline and glucagon, was stimulation of the membrane-bound enzyme adenylate cyclase leading to generation of the second messenger cAMP (cAMP), markedly altered the landscape of receptor biology. Now, the properties of the receptors could be inferred, not from some very downstream physiological event but rather from a very early biochemical readout, cAMP levels. This paradigm was rapidly expanded to the receptors for numerous hormones and neurotransmitters. With the discovery of protein kinase A (PKA) as the immediate downstream effector of cAMP action by Krebs, and of the guanine nucleotide binding proteins that regulate the activity of adenylate cyclase by Rodbell and Gilman, the outline of a concrete biochemical pathway of receptor action had clearly emerged by the mid-1970s. But the almost mythical receptors remained elusive and even controversial.

Many classical pharmacologists of the time remained quite skeptical that the receptors had any real physical reality. Their attitude was typified by Raymond Ahlquist who, using classical pharmacological techniques, in 1948 first proposed the existence of separate α- and β-adrenergic receptors. As late as 1973, he wrote: "This would be true if I were so presumptuous as to believe that α and β receptors really did exist. There are those that think so and even propose to describe their intimate structure. To me they are an abstract concept conceived to explain observed responses of tissues produced by chemicals of various structure" (page 121).[1]

In contrast, biochemists like Sutherland were more comfortable with a molecular basis for drug and hormone action, but still were not ready to accord to the receptors an independent existence. Thus Sutherland wrote in 1967: "It seems likely that in most and perhaps all tissues, the β receptor and adenyl cyclase are the same. The results of many previous studies have pointed to this conclusion, and we feel that the studies with the perfused rat heart have added further to its possible validity" (page 720).[2]

It was within this historical context that my own work on the adrenergic receptors began in the early 1970s. It was motivated by the relatively simple vision that receptors, such as those that activate adenylate cyclase, were membrane proteins, independent of their effectors, which might be studied by appropriate adaptation of the techniques of protein chemistry. The choice of the adrenergic receptors, and most particularly of the β_2-adrenergic receptor (β_2-AR) as my main focus was based on several considerations. An overarching interest in catecholamine action, however, was not one of these. Rather, the choice was based on several practical considerations. As a model system, I wanted a receptor that

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From the Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC.

Correspondence to Robert J. Lefkowitz, MD, Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Box 3821, Duke University Medical Center, Durham, NC 27710. E-mail Lefko001@receptor-biol.duke.edu

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fulfilled several criteria. First, it should be widely distributed in mammalian tissues, play important physiological roles, and in view of my status at the time as a young academic cardiologist, be relevant to cardiovascular biology. Second, since I would clearly need to develop a variety of new experimental approaches, availability of a large array of ligands, both agonists and antagonists, which could be chemically modified for my purposes, was paramount. At the time, this latter requirement virtually eliminated all the receptors for peptide and protein ligands. The adrenergic receptors, however, appeared to fulfill all these criteria.

Ultimately, the success of our endeavor depended on achieving a series of technical breakthroughs that allowed ever closer access to the mysteries of the receptors. In chronological order, these are listed below.

Radioligand Binding: In the early to mid 1970s, we developed radioligands for the direct study, first, of the β-ARs, and then of the α-ARs. Such approaches allowed the properties of the receptors to be measured directly, rather than being inferred from downstream biochemical or physiological readouts. These methods permitted quantitation of the receptors, elucidation of the factors regulating their expression, discovery of the unique actions of guanine nucleotides, and precise investigation and even discovery of new receptor subtypes. They also transformed the way new drug candidates were screened and discovered.

Photoaffinity Labeling Techniques: These were developed to tag the receptors during purification efforts.

Solubilization and Purification: This was a truly daunting challenge because the receptors required 100 000- to 200 000-fold purification from membrane preparations. For comparison, consider the situation with two other contemporaneous objects of study, the nicotinic cholinergic receptor, which was being purified from the electroplax of the electric fish torpedo, and rhodopsin, which was obtained from bovine retina. These proteins each constitute more than 90% of all the protein in purified membrane preparations from the tissues of origin, such that almost no purification was required. The key to our purification efforts was the development of affinity chromatography matrices. These ultimately allowed us to purify to homogeneity all of the 4 known subtypes of adrenergic receptors, the β_1, β_2, α_1, and α_2. Each purified receptor consisted of a single polypeptide that bound ligands with theoretical specific activity for one binding site per receptor, with all the appropriate specificity and stereospecificity characteristics. Still, skepticism persisted as to whether these isolated molecules truly comprised the receptors. These lingering doubts, however, were finally put to rest with the development of reconstitution systems.

Reconstitution Techniques: The isolated β_2-adrenergic receptors were initially transferred via liposomes to the plasma membrane of cells, which lacked the receptors and which accordingly lacked catecholamine-sensitive adenylate cyclase (*Xenopus laevus* erythrocytes). Receptor fusion conveyed responsiveness to isoproterenol (a β-adrenergic agonist) on the previously unresponsive cells. Subsequently, we reconstituted the receptors with various other purified components of the system, culminating in the complete reconstitution of the purified receptor, G_s, and adenylate cyclase into a fully functional isoproterenol responsive system in 1984.

Receptor Cloning: With pure receptors in hand by the early 1980s, our efforts turned to cloning. Despite never having more than about 50 μg of pure receptor at any one time, small stretches of protein sequence were obtained from cyanogen bromide fragments of the receptors, and these were used to design oligonucleotide probes. The rarity of the mRNAs for the receptors complicated initial cDNA cloning attempts for more than a year. Eventually, the first clones for the β_2-AR were pulled from a genomic library. The fortuitous absence of introns in the β_2-adrenergic receptor gene greatly facilitated the completion of its DNA sequence together with our collaborators at Merck Pharmaceuticals.

Our discovery, published in May 1986, that the β_2-AR shared sequence homology and a predicted seven transmembrane spanning architecture with rhodopsin, came as a total surprise to us and everyone else. In spite of the many functional similarities between visual signaling and hormonal activation of adenylate cyclase, it had not been anticipated that the β_2-AR and rhodopsin would share any structural similarity. In fact, the complete amino acid sequence of rhodopsin had been determined several years earlier by conventional protein sequencing, since it was available in such large amounts. However, its seven transmembrane spanning arrangement was analogized only to the prokaryotic light-sensitive protein pump bacteriorhodopsin and was not yet conceived of as relevant to receptor biology.

Over the next several years, we cloned not only the original four adrenergic receptor subtypes but an additional four not previously clearly distinguished by pharmacological criteria, as well as the first "orphan" G protein–coupled receptor, which we subsequently identified as the serotonin 5HT1A receptor. All of these receptors shared sequence similarity and the seven transmembrane spanning organization, as did the muscarinic cholinergic receptor cloned shortly thereafter by Numa's group. Thus, it rapidly became clear, as we had originally speculated in the 1986 study on the cloning of the β_2-AR, that a large family of G protein–coupled seven transmembrane spanning receptors existed. This insight greatly facilitated the cloning of the now huge superfamily of such receptors since this could be done by homology approaches.

Receptor Mutagenesis and Chimeric Receptors: Once the cDNAs for the receptors were available, extensive mutagenesis studies were rapidly accomplished in many laboratories. In addition, we utilized the approach of constructing chimeric receptors between the adenylate cyclase stimulatory β_2 and inhibitory α_2 receptors. When a segment of the β_2-receptor gene, including that portion which encodes the third cytoplasmic loop, was transplanted into the α_2-adrenergic receptor gene, a hybrid receptor resulted which bound ligands with the specificity of the α_2 receptor, but which activated adenylate cyclase through G_s, like the β_2-adrenergic receptor. These results helped shape current concepts of seven transmembrane spanning receptor function in which ligands are bound either by the membrane spanning domains or external loops of the receptors, and G protein

activation occurs through the cytoplasmic loops and carboxy terminal tail.

In the course of our mutagenesis studies, we serendipitously discovered the phenomenon of constitutively active mutant receptors, ie, mutations that render the receptors active even in the absence of agonist. This activity apparently results from the abrogation of intramolecular interactions that normally constrain the receptor to an inactive state. Such mutations were subsequently found to occur spontaneously in humans and to cause an ever growing list of illnesses.

A Universal Mechanism for Receptor Regulation: Contemporaneous with this work were efforts to uncover the molecular basis for the virtually universal phenomenon of desensitization of second messenger signaling, which occurs during persistent stimulation of the receptors. By the early to mid-1980s, we had concluded that the β_2-adrenergic receptor was phosphorylated in association with its desensitization. Two kinases appeared to be active, PKA acting in a classical feedback regulatory fashion, and a cAMP-independent kinase. This latter we named the β-adrenergic receptor kinase or βARK, even in advance of its purification from bovine brain.

A similar story had been evolving for regulation of visual signaling by a molecule called rhodopsin kinase. When we cloned βARK, now known as G protein–coupled receptor kinase 2 (GRK2) and rhodopsin kinase (now known as GRK1) and appreciated their sequence homology, we realized that a gene family of such GRKs existed. Today, this family has seven members (GRK1 to 7). Moreover, the GRKs work in tandem with arrestins, molecules that bind to the GRK-phosphorylated receptors to sterically interfere with their coupling to the G proteins. Four arrestin genes exist in mammals, two of which are limited in expression to the retina and two of which are ubiquitously expressed (β-arrestins 1 and 2, also known as arrestin 2 and 3). These molecules were discovered because in 1987, we were trying to understand why progressive purification of βARK led to loss of its ability to desensitize the β_2-AR in a reconstituted system. When we added back the recently discovered visual arrestin molecule, it restored desensitization, albeit at very high concentrations. Postulating that an analogous molecule must exist in nonvisual tissues, we ultimately cloned β-arrestins 1 and 2 based on their homology with visual arrestin (also known as arrestin 1). Today it is appreciated that this 2-component system of GRKs and arrestins universally regulates seven transmembrane spanning receptors. Moreover, in addition to receptor desensitization, this system mediates their clathrin-mediated internalization, and also links the receptors to a growing number of signaling pathways, such as non–receptor tyrosine kinases and mitogen-activated protein kinases. The β-arrestins thus serve as multifunctional signaling adaptor and scaffold proteins.

In retrospect, the 20-year period from the early 1970s to the early 1990s produced a paradigm shift in the way membrane receptors are viewed and studied. The receptors were transformed from nebulous concepts into discrete molecular entities that could be probed and manipulated to great therapeutic benefit. In the course of this transformation, I was privileged to observe at close range the birth and remarkably rapid growth of this field. I consider myself fortunate indeed, together with my many dozens of devoted and talented students and fellows, to have played a role in all this. We have truly had a magnificent time with the magnificent seven transmembrane spanning receptors.

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