

REVIEW

Seven transmembrane receptors: something old, something new

R. J. Lefkowitz

Departments of Medicine and Biochemistry, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, USA

Received 19 November 2006,
accepted 19 November 2006
Correspondence: R. J. Lefkowitz,
Departments of Medicine and
Biochemistry, Howard Hughes
Medical Institute Duke University
Medical Center, Box 3821 Room
468 CARL Bldg., Research Drive
Durham, NC 27710, USA.
E-mail:
lefk0001@receptor-biol.duke.edu

Abstract

Receptors for hormones, neurotransmitters, drugs, sensory stimuli and many other agents represent the gateway to cellular metabolism and activity. They regulate virtually all physiological processes in mammals. Yet as recently as 40 years ago their very existence was still in question. One class of receptors, those coupled to G proteins (also known as GPCRs or seven transmembrane receptors) comprise by far the largest group (approx. 1000), and are the most important target of clinically used drugs. Here I provide a very personal retrospective of research over the past 35 years which ultimately led to the identification, purification, reconstitution and cloning of the adrenergic receptors; the discovery of their homology with the seven transmembrane spanning visual light receptor rhodopsin and the realization that there was a large gene family of G protein coupled receptors; the elucidation of the molecular mechanisms of receptor desensitization and signalling through G protein-coupled receptor kinases and β -arrestins; and the appreciation that the structure, signalling, and regulatory mechanisms of the receptors are all highly conserved across the large receptor superfamily.

Keywords β -arrestin, adenylate cyclase, cell signalling, desensitization, GPCR, receptor.

Early years

I have been fascinated by receptors throughout my scientific career. But the fascination of biologists with receptors dates back more than a century to classical work by Ehrlich on interaction of antigens with cells, and then to Langley and Dale with nicotinic cholinergic and adrenergic systems in the earliest years of the 20th century (Limbird 1996). Subsequently, physiological techniques were used to study receptors for decades such that by the mid-20th century, a body of now classical receptor theory had been developed, derived largely from the application of the principle of mass action to pharmacological dose response curves. During the 1960s and early 1970s biochemists joined the enterprise, and began to flesh out the molecular steps which lay between the putative receptors and the ultimate response elements within the cells. This led in succession to the discovery of second messenger generating enzymes such as adenylate cyclase

and phospholipase C, which produce messengers such as cyclic adenosine monophosphate (cAMP), diacylglycerol and IP₃; the second messenger dependent kinases, such as protein kinase A (PKA) and protein kinase C (PKC); and heterotrimeric G proteins which couple the receptors to the second messenger generating enzymes (Gilman 1987). The significance of these discoveries lies in the fact that they brought the measured consequences of receptor stimulation closer and closer to the receptors themselves. Thus, it was no longer necessary to measure complex physiological responses such as the twitching of a muscle preparation or secretion from a gland which were many steps downstream of the receptors, but rather it was now possible to measure much more proximal events such as the generation of a second messenger.

However, even into the 1970s the receptors themselves remained elusive. In fact, a considerable body of opinion held that receptors, as we now understand them, did not exist as discrete molecular entities.

Exemplary of this skepticism is the following quotation from the early 1970s by the distinguished American pharmacologist Raymond Ahlquist who, ironically, some 25 years before had pioneered the concept of distinct α and β -adrenergic receptors for catecholamines. 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’ (Ahlquist 1973).

As a biochemist, Earl Sutherland was more accepting of a molecular ‘receptor’, but even he apparently did not think of it as an independent entity but rather as some sort of binding site on the enzyme adenylate cyclase. Thus, in 1967 he wrote of the β -adrenergic receptor for catecholamines; ‘...It seems likely that in most and perhaps all tissues the beta receptor and adenylyl 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’ (Sutherland 1967).

The molecular era-radioligand binding

So it was against this background of significant skepticism as to the existence of receptors that work began in the very early 1970s to ‘bring them to life’ as discrete molecular entities representing the portal of entry to the cellular signalling machinery. As a young academic cardiologist I chose the adrenergic receptors, and particularly the β_2 -adrenergic receptor, as my initial model for study. This offered both pragmatic and somewhat more personal benefits. At a pragmatic level, there were many dozens of adrenergic ligands available which I felt would be useful in developing the new tools which would be necessary for the endeavour, such as radioligands, photoaffinity labels, affinity chromatography resins, etc. At a more personal level, I wanted to study a class of receptors with obvious relevance to cardiovascular disease.

As is often the case, the key to moving forward was the development of novel technologies, the absence of which had previously stymied progress. The initial technique that was needed was a means of identifying and studying the receptors directly by radioligand binding so that their properties no longer needed to be inferred from downstream signalling events. We were successful in developing such radioligand binding methods, initially for the β -adrenergic receptors, and then for the α -adrenergic receptors (Mukherjee *et al.* 1975). Contemporaneously, other laboratories were developing ligands for other receptors such as for example the opioid, glucagon and muscarinic

cholinergic receptors (Rodbell *et al.* 1971, Pert & Snyder 1973, Yamamura & Snyder 1974). With the development of these methods in the early 1970s the era of molecular studies of cellular plasma membrane receptors was born. These new approaches led to rapid progress in understanding the dynamic regulation of receptors by all manner of physiological and pathophysiological circumstances; rapidly expanded the number of receptor subtypes which were appreciated; and were also useful for developing and exploring new theories of receptor action. For example, the unique effect of guanine nucleotides on agonist, but not antagonist binding competition displacement curves, and the appreciation that agonists could distinguish interconvertible high- and low-affinity states of the receptors which antagonists could not, led to development of the widely applied ‘ternary complex model’ for the β -adrenergic receptors. This model was found to be widely applicable to the family of G protein coupled receptors and provided a simple way of quantitating coupling efficiency of the receptors to the recently discovered G proteins. It also provided a conceptual framework for understanding how agonist stabilization of a high affinity complex of receptor and G protein could lead to activation of effectors such as adenylate cyclase (De Lean *et al.* 1980).

Purification of the receptors

Another important consequence of the development of radioligand binding techniques was that they permitted, for the first time, an approach to isolation and characterization of the receptors. At the time, there were three objects of study which, in retrospect, were relevant to the overall goal of understanding the structure of receptors. These were the nicotinic cholinergic receptor present in the electric organs of electric fish such as *torpedo*; the visual pigment rhodopsin, present in rod outer segments; and the β_2 -adrenergic receptor. Interestingly, back then, no one conceived of rhodopsin as a receptor and the work in that area was not closely followed by those pursuing G protein coupled receptors. Unlike the situation for the β_2 -adrenergic receptors, highly enriched sources of nicotinic cholinergic receptors and rhodopsin were available. Thus, membranes derived from the electroplax of *torpedo* or from bovine rod outer segments consisted almost entirely of nicotinic cholinergic receptors or rhodopsin respectively. In contrast, β_2 -adrenergic receptors and essentially all other G protein coupled receptors are almost trace contaminants of cell membranes, generally being present at a concentration in the range of 20–100 fmol mg⁻¹ protein and thus requiring several hundred thousand-fold purification.

The key element in our ultimate success was that we were able to develop affinity chromatography matrices which allowed us to purify all four of the then known subtypes of adrenergic receptors, the β_2 and β_1 , and the α_2 and α_1 -adrenergic receptors. In each case, we covalently linked α or β -adrenergic antagonists through spacer arms to Sepharose beads. To these columns, we applied solubilized preparations of plasma membranes derived from various sources which could be shown to contain either the β - or α -receptors by radioligand binding. Solubilization of the receptors in a functionally active form was in itself a major challenge, since almost every detergent we tried seemed to inactivate ligand binding. Only the plant glycoside digitonin yielded soluble receptor in an active form. By combining affinity chromatography with biospecific adsorption and elution by specific adrenergic counter ligands, with more conventional chromatographic approaches, we ultimately succeeded in purifying the four subtypes of adrenergic receptor. This effort consumed more than a dozen years of intense effort by many devoted students and fellows. Each of the adrenergic receptor subtypes was shown to consist of a single polypeptide chain, of approximate molecular weight 60–65 000 Da. All were glycoproteins which bound appropriate radioligands with exactly the specificity and stereospecificity which would be predicted based on pharmacological data derived from *in vivo* experiments (Dohlman *et al.* 1991).

Receptor reconstitution

With the successful purification of the receptors I had imagined we would put to rest the prevailing skepticism surrounding their existence. However, this was not the case. Only when we successfully reconstituted the receptors and showed that they were able to convey catecholamine responsiveness to previously unresponsive systems was it fully accepted that the isolated molecules were in fact the genuine receptors. This we accomplished in two ways. Initially, we reconstituted the purified β_2 -adrenergic receptors in phospholipid vesicles and fused them with *Xenopus laevis* erythrocytes which, while containing no β -adrenergic receptors, do contain the adenylate cyclase system and other receptors such as for example those for prostaglandins. The adenylate cyclase in these cells is not normally responsive to β -adrenergic agonists but after incorporation of our receptor proteins into the membranes of the cells, β -adrenergic responsiveness was acquired (Cerione *et al.* 1983). A year later we succeeded, in collaboration with the laboratories of Lutz Birnbaumer and Eva Neer, in reconstituting the purified receptors together with purified heterotrimeric Gs and the catalytic moiety of adenylate cyclase. Not only did these

studies further validate that the isolated proteins were in fact the receptors, but they demonstrated that only these three proteins are necessary to form a hormone responsive adenylate cyclase signalling system (Cerione *et al.* 1984).

Cloning of the receptor genes and cDNAs

We next turned our attention to cloning the receptor genes and cDNAs to decipher their primary structure. We focused initially on the β_2 -adrenergic receptors. With purified protein in hand (never more than about 50 μ g at any one time), we were able to obtain the amino acid sequences of five cyanogen bromide peptides derived from the receptor. Using these sequences as a basis for the design of oligonucleotide probes, we spent the next several years working to clone the receptors. This phase of the work was done collaboratively between my laboratory and a group at Merck. After many missteps and setbacks, we ultimately succeeded in deducing the primary sequence of the receptor by cloning its gene from a genomic library. This was possible because, remarkably, the gene turned out to be intronless, one of only a very small handful of genes known at the time which lacked introns (Dixon *et al.* 1986).

The sequence of the receptor revealed a remarkable surprise, the presence of seven transmembrane domains with sequence homology to rhodopsin apparent within several of the membrane spans. Today, it is hard to understand why this discovery 20 years ago was so surprising. All of us in the signalling community at the time were quite aware of the obvious functional analogies between rhodopsin, its G protein transducer, and the cGMP phosphodiesterase on the one hand and the β_2 -adrenergic receptor, G protein, adenylate cyclase system on the other. Still, no one at that time expected that there would be any *structural* relationship between the receptors and rhodopsin. In fact, the sequence of rhodopsin had been determined several years before, not by cloning, but rather by classical protein sequencing and Edman degradation (Ovchinnikov 1982). This was possible because of the extremely large quantities of protein which were available. When it was found that its sequence predicted seven membrane-spans it was immediately analogized with the only other seven membrane spanning protein known at the time, bacteriorhodopsin (Ovchinnikov 1982). This is a light sensitive proton pump found in archaeobacteria. It was concluded that seven membrane spans must be a signature feature of the structure of light sensitive proteins. Only with the cloning of the β_2 -adrenergic receptor did it immediately become clear that in fact it might be the signature structural feature of all G protein coupled receptors (Dixon *et al.* 1986).

Rapidly, we were able to confirm this idea by cloning the α_{2A} -adrenergic receptor the next year (Kobilka *et al.* 1987). Over the next few years, we eventually cloned a total of eight adrenergic receptors (Dohlman *et al.* 1991). All contained the now classical seven membrane spanning domain arrangement as well as other features initially discerned in the β_2 -adrenergic receptor sequence. These include consensus sites for N-linked glycosylation near the amino terminus and sites for regulatory phosphorylation on the inner loops and especially the carboxy terminal tail. The cloning of the first muscarinic cholinergic receptor (Kubo *et al.* 1986) and substance K receptor (Masu *et al.* 1987) further confirmed this notion. Over the next few years the family of seven transmembrane receptors expanded rapidly as more and more of the receptors were cloned, almost invariably by homology approaches such as low stringency screening or PCR primers (Dohlman *et al.* 1991).

Orphan receptors

Not all of the receptors which were cloned were of known function. Those cases in which a sequence for a seven membrane spanning receptor was in hand but either the ligand or the function or both were unknown were called 'orphan' receptors. We cloned the first orphan receptor within months of cloning the β_2 -adrenergic receptor. In performing low stringency Southern blots with human genomic DNA using the β_2 -adrenergic receptor cDNA as a probe, we found that, of the multiple bands observed, one was considerably more prominent than all the others. Reasoning that this must be the very closely related β_1 -adrenergic receptor, we cloned it from a size-selected library. In fact, the gene did have all the characteristics of a seven membrane spanning receptor, but when expressed in xenopus oocytes, it did not bind β_1 -adrenergic receptor ligands. It thus became the first orphan receptor. Within a year or so we were able to decipher its true identity, which was the 5HT_{1A} serotonin receptor, the first serotonin receptor to be cloned (Fargis *et al.* 1988). Some years later, in 1991, Buck and Axel, using degenerate PCR primers based on the dozen or so receptors which by then had been cloned, were able to clone a large family of olfactory receptors numbering almost 500 (Buck & Axel 1991). Most of these are still orphans, in the sense that the specific odorants with which they interact are not clearly delineated. Almost a decade after this, in 1999, Zucker *et al.* and others cloned a family of taste receptors (Hoon *et al.* 1999). Although many of these were initially orphans, their functions in perceiving bitter, sweet and other flavors such as umami have since been deciphered (Zhao *et al.* 2003). Today, it is appreciated that the

superfamily of seven transmembrane receptors represents one of the largest gene families in the mammalian genome, numbering close to a thousand (Frederiksson & Schiöth 2005). Of the 500 or so non-olfactory receptors, perhaps as many as half currently remain orphans, presenting a remarkable therapeutic opportunity for the development of novel drugs which can target these receptors.

Receptor structure and function

Once the primary structure of the receptors was understood, efforts turned toward trying to understand how this highly conserved structure was able to mediate the two classical functions of a receptor, namely, binding specific ligands and activation of specific effectors. In our work, we utilized two main approaches, classical site-directed mutagenesis and construction of chimaeric receptors. The first chimeras which we created contained sequences derived from various portions of the α_{2a} and β_2 -adrenergic receptors. These two receptors represented a very attractive pair for creating and studying such chimaeric receptors. Their sequences were quite homologous, about 50% in the membrane spans, and they both bound adrenergic ligands, albeit with distinct specificities. However, they had opposite biochemical and physiological effects. Thus, β_2 -adrenergic receptors classically activate adenylate cyclase through G_s, whereas the α_2 -receptors inhibit the enzyme through G_i. By 'stitching together' sections of the two receptors we created about a dozen different chimaeric molecules (Kobilka *et al.* 1988). By studying their properties, we were able to demonstrate that the ligand binding specificity was largely determined by residues in the membrane spans, whereas the specificity of coupling to G_s vs. G_i was determined by sequences present primarily in the amino and carboxyl terminal portions of the third intracellular loop. Much additional work by our group and many other groups over the years have further refined this understanding. Thus, we now know that sequences in various intracellular loops of different receptors determine G protein interaction specificity. In general, it is those intracellular sequences which are in closest apposition to the plasma membrane which appear to be most crucially involved (Ostrowski *et al.* 1992). Depending on the receptor, ligand binding may be determined by sequences in the extracellular loops and/or the amino terminus, depending on which class of ligand (small molecule, large glycoprotein hormone, etc.), is involved. The spectrum of ligands which interact with the family of seven transmembrane receptors is extraordinary, ranging from ions to small molecules to very large protein and glycoprotein ligands, amongst many others.

Constitutively active mutant receptors and inverse agonists

A serendipitous discovery which flowed from our attempts to understand which regions of the receptors were involved in coupling to G proteins, was the discovery of so-called 'constitutively active mutant receptors'. These are receptors which are active even in the absence of agonist occupancy. In the course of making mutations in the distal part of the third intracellular loop of the α_{1B} -adrenergic receptor which were expected to lead to loss of signalling functions, we discovered quite unexpectedly that some of these mutations actually led to receptors which were active even in the absence of agonists, i.e. they were 'constitutively active' (Cotecchia *et al.* 1990). Additional work led to the conclusion that these mutations abrogate certain key intra-receptor interactions which normally constrain the receptor to the inactive state, thereby allowing it to 'relax' into an active conformation which apparently mimics that induced by agonist occupancy. Subsequently, it was discovered that many receptors undergo spontaneous mutations which lead to such constitutive activity and that this can actually lead to a variety of human illnesses. These include mutations in the TSH receptor which lead to 'hot thyroid nodules', which are thyroid adenomas which secrete thyroid hormone, as well as certain forms of precocious puberty in males, who inherit a gene for a constitutively active LH receptor. About a dozen such diseases due to constitutively activating mutations in 7TM receptors have been described (Spiegel 1998). A further interesting development was the discovery that some antagonists are actually able to inhibit the activity of these constitutively active mutant receptors, i.e. they are actually able to stabilize the inactive state of the receptor. Such antagonists are generally referred to as 'inverse agonists' and can be distinguished from classical neutral antagonists which, while blocking the receptors, are not able to lower their activity (Bond *et al.* 1995).

A universal regulatory mechanism

Contemporaneous with our work on the structure of the receptors was our attempt to understand the mechanisms by which the receptors were regulated. This effort, which stemmed from the earliest years of my research career, was based on my sense that the ubiquitous phenomenon of receptor desensitization was a classic example of perhaps the most pervasive principle of physiology, homeostasis. Thus, whenever our laboratory developed some new technique for studying the receptors I was always anxious to apply it immediately to the desensitization problem. This

work ultimately led to the discovery that two small families of proteins appear to universally regulate essentially all of the seven transmembrane receptors (Pitcher *et al.* 1998, Shenoy & Lefkowitz 2003). The proteins share, in common with heterotrimeric G proteins, the ability to interact virtually universally with all of the receptors in a strictly stimulus dependent fashion. These two families of regulatory proteins are the β -arrestins and the G protein coupled receptor kinases (GRKs). Within seconds of a receptor being activated and interacting with its G protein it begins to be phosphorylated by one of the GRKs. This phosphorylation occurs largely on the carboxyl terminal cytoplasmic tail but can also occur on other intracellular domains. The phosphorylation promotes the interaction of β -arrestins with the phosphorylated receptors, leading in turn to desensitization of further G protein signalling by steric exclusion by the β -arrestins.

The G protein coupled receptor kinases

We initially discovered the GRKs when, in about 1980, we applied some of our recently developed photoaffinity labelling techniques for the β -adrenergic receptors to the problem of desensitization. We exposed cells to a β -adrenergic agonist isoproterenol for a few minutes, thereby desensitizing them to further stimulation. We then photoaffinity labelled the receptors from naive and desensitized cells and examined the mobility of the labelled receptors on SDS polyacrylamide gels. We found that the mobility of the desensitized receptors was retarded (Stadel *et al.* 1982). This suggested some covalent modification such as phosphorylation. Labelling the cells with $^{32}\text{P}_i$ quickly confirmed that the desensitized receptors were in fact being phosphorylated (Stadel *et al.* 1983). Over the next decade, we worked to identify the enzyme that was mediating this phosphorylation and discovered that it was a previously unknown member of the kinase family, which we initially named β ARK (β -adrenergic receptor kinase). Today the enzyme is known as GRK2. We were able to purify the enzyme from bovine brain and then to clone its cDNA (Benovic *et al.* 1989).

Contemporaneous with this work, others had been studying an enzyme present exclusively in the retina which phosphorylated rhodopsin in a strictly light-dependent fashion and which appeared to contribute to its deactivation. The enzyme was called rhodopsin kinase and had obvious functional analogies with β ARK (Wilden & Kuhn 1982). Accordingly, we purified the enzyme and cloned its cDNA and found that it was a member of the same new gene family founded by β ARK (Lorenz *et al.* 1991). Over the next few years, we and others cloned additional GRKs and today we know that there are seven members of the family (Pitcher *et al.*

1998). GRK1 and 7 are found exclusively in retinal rods and cones, respectively, where they regulate rhodopsin and the colour opsins. Two other subfamilies of GRKs have been delineated, consisting of GRK2 and 3 on the one hand and GRKs 4, 5 and 6 on the other. GRKs 2, 3, 5 and 6 are quite ubiquitously distributed. All the enzymes consist of a tripartite domain organization with a central catalytic domain flanked by two regulatory domains. GRKs 2 and 3 contain a carboxyl terminal PH domain which interacts with inositol phospholipids and $G\beta\gamma$ -released by stimulation of heterotrimeric G proteins. Interaction of these two ligands with the PH domain translocates these GRKs to the membrane bound receptors. GRKs 5 and 6 are constitutively associated with the membrane through various mechanisms.

The β -arrestins

Discovery of the β -arrestins flowed directly from the work on β ARK. In the course of purifying the enzyme we discovered, much to our initial chagrin, that the more we purified it, the more it seemed to lose its ability to desensitize the β_2 -adrenergic receptor, as assessed in a reconstituted system of receptor and G protein in phospholipid vesicles. This suggested that perhaps we were losing some necessary co-factor for the desensitization during the preparation of β -ARK. Just about this time (1987), Herman Kuhn reported that an abundant retinal protein which previously had been known as either '48K protein' or 'S antigen' was somehow able to work with rhodopsin kinase to deactivate rhodopsin (Kuhn & Wilden 1987). Observations such as this led to its being renamed thereafter as arrestin. We reasoned that what we were losing during our β -ARK preparations might be a molecule which was functionally analogous to S antigen. That it was not S antigen itself was clear, since this protein was known to be expressed only in the retina and perhaps to some extent in the pineal gland. Accordingly, I contacted Kuhn and obtained some of his authentic 48K protein. We were then able to demonstrate, in our reconstitution experiments, that visual 48 K protein (aka arrestin), was in fact able to restore to highly purified β -ARK preparations the ability to desensitize the receptor with respect to G protein activation. However, this required very high concentrations of visual arrestin, generally present at >100 : 1 molar excess over G protein (Benovic *et al.* 1987).

Later that year, Shinohara cloned the cDNA for S antigen (Shinohara *et al.* 1987). Reasoning that the molecule we were losing during our purifications might be not only functionally, but also structurally, analogous to the visual molecule, we obtained Shinohara's clone and used it for low stringency screening. We were

able to obtain clones for a homologous molecule which we named β -arrestin (Lohse *et al.* 1990), and within another year or two, for a second molecule we called β -arrestin2 (Attramadal *et al.* 1992). Today these molecules are also referred to as arrestins 2 and 3, respectively. With authentic recombinant visual arrestin, β -arrestin1 and β -arrestin2 in hand, we were able to compare their abilities to desensitize β ARK phosphorylated β_2 -adrenergic receptors, or rhodopsin kinase phosphorylated rhodopsin, in reconstituted systems. In such systems we could demonstrate the great specificity of the β -arrestins for the beta receptor over rhodopsin and vice-versa for visual arrestin. In the reconstituted β_2 -adrenergic receptor system, β -arrestins 1 and 2 demonstrated very significant inhibition of coupling of receptor to G protein at molar ratios with G protein of 1 : 1 (Lohse *et al.* 1990, Attramadal *et al.* 1992). Today we know there are four arrestin genes: arrestin1 and x-arrestin, expressed only in retinal rods and cones, respectively, and the ubiquitously expressed β -arrestins 1 and 2. All the molecules share a highly conserved structure consisting of two domains, each composed largely of anti-parallel beta sheets (Hirsch *et al.* 1999).

New aspects of β -arrestin function

As I have just described, the GRKs and the arrestins were discovered in the context of trying to unravel the mechanisms which desensitize G protein mediated signalling. Over the past 10 years, there have been a series of discoveries which have revealed a whole new array of functions carried out by this versatile system. These new functions relate to the ability of β -arrestins to serve as adaptors or scaffolds for various molecules involved in both clathrin-mediated endocytosis of the receptors as well as in signalling functions. Even the desensitization function of the β -arrestins has turned out to be more complicated and, in a sense, more elegant than initially appreciated. Thus, we now know that the β -arrestins, in addition to their ability to inhibit the rate of cyclic AMP synthesis by blocking activation of Gs, are also able to increase its rate of degradation. They accomplish this by recruiting phosphodiesterases such as PDE4D to the receptor, thus placing them in close proximity to the sites of generation of the second messenger (Perry *et al.* 2002). Thus, the β -arrestins act to desensitize cyclic AMP formation and PKA activation by a concerted mechanism in which they both impede the rate of its synthesis and enhance the rate of its degradation.

We now also know that β -arrestins are able to serve as adaptors which link the agonist stimulated receptors to a growing list of components of the endocytic machinery including, for example, clathrin, the clathrin adaptor AP2, the small G protein ARF6 and its guanine

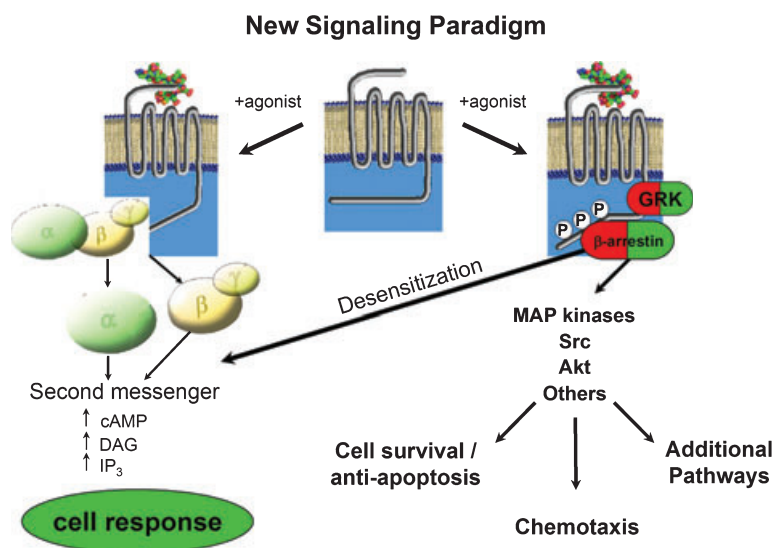
nucleotide exchange factor ARNO as well as NSF (Claing *et al.* 2002). In so doing they mediate receptor internalization. A further interesting feature is that the β -arrestins bind the E3 ubiquitin ligase MDM2, which ubiquitinates the β -arrestin only if β -arrestin is interacting with a stimulated receptor. This agonist-stimulated ubiquitination of β -arrestin is required for it to perform its endocytic functions (Shenoy *et al.* 2001). The β -arrestins also serve as adaptors which bring MDM2 and other E3 ligases into proximity with the receptors (Shenoy *et al.* 2001, Girnita *et al.* 2005). Agonist stimulated receptor ubiquitination is not involved in the internalization process but rather is prerequisite for targeting the internalized receptors to lysosomes for degradation (Shenoy *et al.* 2001).

The newest set of discoveries relate to the recently appreciated fact that the β -arrestins are able to serve as signal transducers in their own right (Lefkowitz & Shenoy 2005). We discovered this when we found that β -arrestins were able to bind c-Src and thereby bring it into complex with the receptors. This serves to facilitate Src-dependent, receptor-stimulated activation of effectors such as the MAP kinases (Lefkowitz & Shenoy 2005). Subsequently, we have found that β -arrestins are able to scaffold various MAP kinase pathways including ERK1/2, P38 and JNK3. Over the past 5 years, a rapidly growing list of signalling pathways has been found to be stimulated by β -arrestin-mediated signal transduction from the receptors. In addition to those mentioned above, these include pathways such as AKT, PI3 kinase and others. Thus, as shown in Figure 1, our understanding of signal transduction regulation by 7TM receptors has undergone a major revision in the past few years. The change is that the β -arrestin/GRK system is now appreciated to function not solely as a desensitization mechanism, but simultaneously as a

signal transduction mechanism. Thus the β -arrestin/GRK system is truly bifunctional. While the physiological consequences of this signal transduction are just being uncovered now, we already know that signals transduced via β -arrestins mediate cell survival and anti-apoptotic effects, some forms of chemotaxis, a novel pathway for dopaminergic receptor control of behaviours and cardiac contractility. This list is sure to grow in the coming years (Lefkowitz & Shenoy 2005).

Over the past several years, we have utilized the angiotensin receptor system as a model for understanding β -arrestin mediated signalling. We have utilized a mutated angiotensin octapeptide referred to as SII angiotensin. The molecule contains three substitutions, sarcosine at position one, and isoleucine at positions 4 and 8. In confirmation of data previously published by others (Holloway *et al.* 2002), we found that SII angiotensin is unable to stimulate G protein-mediated effects through the angiotensin II 1A receptors such as PI production, calcium mobilization, and GTP γ S binding to G proteins (Wei *et al.* 2003). However, the peptide ligand is able to stimulate β -arrestin recruitment to the receptors and β -arrestin-mediated endocytosis of the receptors (Wei *et al.* 2003). These findings suggested that this ligand might be able to selectively activate β -arrestin-mediated signalling, although it is unable to stimulate G protein mediated signalling. In fact, in HEK293 cells, we are able to demonstrate that this is precisely the case (Fig. 2). β -Arrestin2 but not β -arrestin1 mediates this signalling (Ahn *et al.* 2004b). When β -arrestin2 levels in the cells are reduced by siRNA the β -arrestin mediated signalling is lost and only G protein mediated signalling can be observed. As shown in the figure, this G protein mediated activation of ERK is quite transient. It is rapidly turned on and rapidly turned off by receptor desensitization. By

Figure 1 β -Arrestin bifunctionality as a new paradigm for 7TM receptor signalling. The binding of agonist to its cognate receptor initiates not only classical G protein-mediated signalling via second messengers, but also GRK and β -arrestin dependent signals. New pathways with β -arrestin activating proteins such as ERK and Src affect cell responses including anti-apoptotic signalling, cardiac contractility, and dopaminergic behaviours among others. These roles are in addition to the historically defined roles for the GRKs and β -arrestins in 7TM receptor desensitization and internalization.



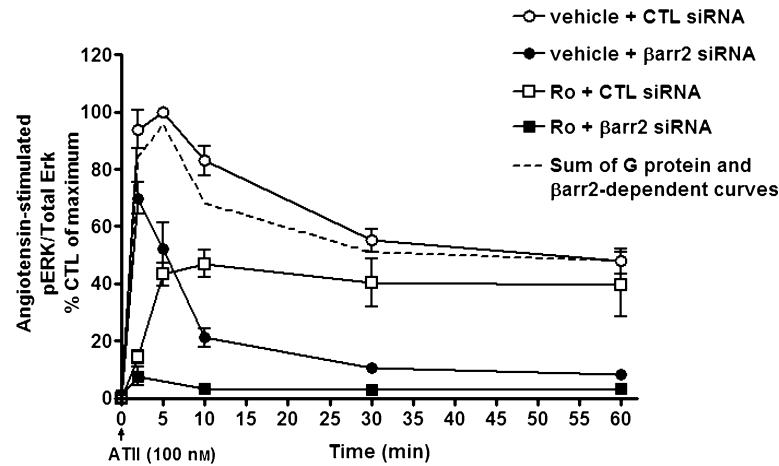


Figure 2 G protein- and β -arrestin-mediated ERK activation defined by temporal patterns. Angiotensin II in HEK293 cells overexpressing AT_{1A} receptor stimulates ERK1/2 phosphorylation through both G protein- and β -arrestin-dependent pathways (open circles). Transfection of cells with siRNA specific for β -arrestin2 reveals the G-protein-dependent ERK activity: a transient pool of pERK peaking at 2 min (closed circles). The PKC inhibitor Ro-31-8425 blocks this G protein-dependent pathway of ERK activation and exposes a prolonged response representing β -arrestin-mediated ERK signalling (open squares). Combining β -arrestin 2 siRNA transfection with Ro-31-8425 effectively eliminates angiotensin II ERK stimulation (closed squares) suggesting these two pathways comprise the entirety of ERK signalling in this system. This conclusion is supported by the observation that a mathematical sum of the isolated G protein- and β -arrestin-dependent pERK curves (dashed line) replicates the dynamics of angiotensin signalling in these cells [data are modified from Ahn *et al.* (2004a) with permission from the *Journal of Biological Chemistry*.]

contrast, in the presence of PKC inhibitors which block the G protein mediated component of ERK activation, only β -arrestin mediated signalling is observed. As can be observed in the figure, this is slower in onset but much more protracted than the G protein signalling. In the presence of both the PKC inhibitor and siRNA for β -arrestin2 all ERK activation is blocked, indicating that in this system G protein dependent and β -arrestin dependent signalling pathways account for all the ERK activation by the angiotensin receptor (Ahn *et al.* 2004a). Entirely analogous results have now been found for at least half a dozen receptors which activate Gq and Gs dependent signalling. In the case of receptors which activate ERK through Gi, β -arrestin dependent signalling has also been observed but in this case, the β -arrestin and Gi dependent mechanisms appear to operate in a cooperative fashion (Sun *et al.* 2002). The molecular basis for this, however, is unknown.

Another striking difference between the β -arrestin dependent and G protein dependent pathways leading to ERK activation is the ultimate subcellular destination of the activated ERK. In the case of the G protein dependent pathway, activated ERK is found diffusely through the cytosol and the nucleus of the cell as revealed by immunocytochemistry with antibodies which recognize only the activated phosphorylated form of ERK (Ahn *et al.* 2004a). The nuclear ERK appears to phosphorylate transcription factors which then regulate diverse programmes of gene transcription. These are the classical functions initially described

for the MAP kinases. In contrast, ERK activated via β -arrestin2 is specifically excluded from the nucleus and appears to be concentrated in the cytosol in endocytic vesicles where it co-localizes with β -arrestin and the receptors (Ahn *et al.* 2004a). While the relevant cytosolic ERK substrates phosphorylated by this pool of activated ERK are not yet known with clarity, there is growing evidence that these relate to regulation of the actin cytoskeleton, changes in cell shape, motility and chemotaxis amongst other processes.

Therapeutic possibilities

The new understanding that the β -arrestin and GRK system is bifunctional and able to independently activate signalling pathways while simultaneously desensitizing G protein dependent signalling may have significant implications for the development of a novel class therapeutic agents. The basis for this hypothesis is outlined in Figure 3. As shown in Figure 3a, activation of the 7TM receptor by an agonist such as angiotensin leads to interaction of the activated receptor with the G protein leading to 'classical' signalling processes. Next, the receptor interacts with GRKs and β -arrestin leading to desensitization of G protein signalling as well as β -arrestin-mediated signalling (Fig. 3b). Figure 3c indicates that it is possible to develop ligands which can uniquely activate the β -arrestin dependent pathways without activating the G protein dependent pathways. SII angiotensin would be an example of such a ligand. In

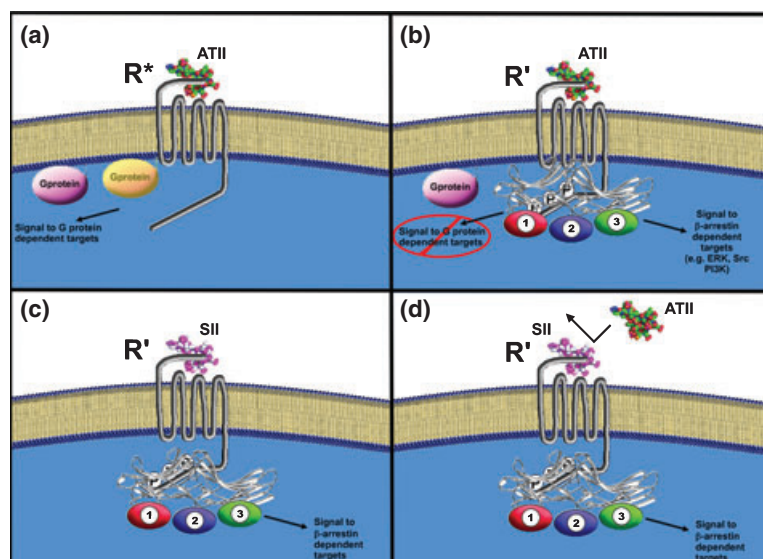


Figure 3 Therapeutic possibilities of biased 7TM receptor ligands. (a). In the traditional view of receptor signalling, interaction of the 7TM receptor and ligand signals to heterotrimeric G proteins, initiating second messenger production, and activating second messenger kinases (i.e. PKA and PKC). (b) Phosphorylation of the agonist-occupied 7TM receptor by the GRKs leads to β -arrestin recruitment and inhibition of G protein signals by homologous desensitization as well as activation of novel β -arrestin-scaffolded signalling pathways (represented by the sequentially numbered balls bound to the β -arrestin). (c) Biased agonists selectively engage either G protein- or β -arrestin-mediated signalling. For example, SII angiotensin acting on the AT₁R does not engage G_{q/11} but does induce β -arrestin recruitment and G protein-independent activation of ERK. (d) Administration of a biased agonist such as SII could have valuable therapeutic possibilities. In addition to activating selective intracellular signalling pathways these ligands also serve as antagonists to the receptor's natural ligand, effectively acting as a 'super-receptor blocker'. R* and R' represent distinct active conformations of the receptor.

the current parlance of pharmacologists, such ligands are referred to as 'biased agonists'. An interesting implication of these findings is that the classical conception of receptors as existing in two states—inactive and active, must now be discarded. Clearly there are multiple active states of the receptor, at minimum two, and probably more. Thus, the conformation of the active receptor stabilized by a ligand such as SII angiotensin, which can activate β -arrestin but not G protein, must be different than that stabilized by angiotensin itself, which can activate both pathways.

These findings suggest the possibility of a new type of therapeutic agent as is illustrated by Figure 3d. Agents such as SII angiotensin, while agonists for β -arrestin mediated signalling pathways, are simultaneously antagonists for the G protein dependent signalling pathways. This is true because they competitively occupy the same ligand binding site on the receptor. To illustrate why this may open the way to the development of a novel class of 'super-receptor blockers' let us consider the examples of the classical β -adrenergic antagonists and the angiotensin receptor blockers (ARBs). These two types of drugs are amongst the most widely prescribed in the world for the treatment of a variety of cardiovascular illnesses inclu-

ding congestive heart failure, angina and hypertension. Their effectiveness is based on the fact that they are able to block the deleterious G protein mediated effects of catecholamines and angiotensin on the cardiovascular system. Consider the possible effects of a beta blocker or an angiotensin receptor blocker which, like conventional blockers, is able to antagonize G protein dependent signalling, but which at the same time is also able to engage potentially salutary β -arrestin mediated signalling pathways. As shown in the diagram, pathways such as ERK, PI3 kinase and AKT are all known to be stimulated through β -arrestin mediated mechanisms. All of these are pro-survival and anti-apoptotic, which in the setting of cardiovascular diseases may be very positive. Thus, such a 'super beta blocker' or 'super ARB' might have potential benefits beyond those of the conventional blockers which, while they also antagonize deleterious G protein mediated signalling, do not engage β -arrestin mediated pathways.

Conflict of interest

None.

The author wishes to thank Chris Nelson for editorial assistance and Elizabeth Hall for typing the manuscript.

References

- Ahlquist, R.P. 1973. Adrenergic receptors: a personal and practical view. *Perspect Biol Med* 17, 119–122.
- Ahn, S., Shenoy, S.K., Wei, H. & Lefkowitz, R.J. 2004a. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279, 35 518–35 525.
- Ahn, S., Wei, H., Garrison, T.R. & Lefkowitz, R.J. 2004b. Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* 279, 7807–7811.
- Attramadal, H., Arriza, J.L., Aoki, C. *et al.* 1992. β -arrestin2-a novel member of the arrestin/ β -arrestin gene family. *J Biol Chem* 267, 17 882–17 890.
- Benovic, J.L., Kuhn, H., Weyand, I., Codina, J., Caron, M.G. & Lefkowitz, R.J. 1987. Functional desensitization of the isolated β -adrenergic receptor by the β -adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proc Natl Acad Sci USA* 84, 8879–8882.
- Benovic, J.L., DeBlasi, A., Stone, W.C., Caron, M.G. & Lefkowitz, R.J. 1989. β -adrenergic receptor kinase: Primary structure delineates a multigene family. *Science* 246, 235–240.
- Bond, R.A., Leff, P., Johnson, T.D. *et al.* 1995. Physiologic effects of inverse agonists in transgenic mice with myocardial overexpression of β_2 -adrenoceptor. *Nature* 374, 272–276.
- Buck, L. & Axel, R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65, 175–187.
- Cerione, R.A., Sibley, D.R., Codina, J. *et al.* 1984. Reconstitution of a hormone-sensitive adenylate cyclase system. The pure beta-adrenergic receptor and guanine nucleotide regulatory protein confer hormone responsiveness on the resolved catalytic unit. *J Biol Chem* 259, 9979–9982.
- Cerione, R.A., Strulovici, B., Benovic, J.L., Lefkowitz, R.J. & Caron, M.G. 1983. Pure beta-adrenergic receptor: the single polypeptide confers catecholamine responsiveness to adenylate cyclase. *Nature* 306, 562–566.
- Claing, A., Laporte, S.A., Caron, M.D. & Lefkowitz, R.J. (2002). Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol* 66, 61–79.
- Cotecchia, S., Exum, S., Caron, M.G. & Lefkowitz, R.J. 1990. Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc Natl Acad Sci USA* 87, 2896–2900.
- De Lean, A., Stadel, J.M. & Lefkowitz, R.J. 1980. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β -adrenergic receptor. *J Biol Chem* 255, 7108–7117.
- Dixon, R.A.F., Kobilka, B.K., Strader, D.J. *et al.* 1986. Cloning of the gene and cDNA for mammalian β -adrenergic receptor and homology with rhodopsin. *Nature* 321, 75–79.
- Dohlman, H.G., Thorner, J., Caron, M.G. & Lefkowitz, R.J. 1991. Model systems for the study of seven-transmembrane-segment receptors. *Annu Rev Biochem* 60, 653–688.
- Fargin, A., Raymond, J. R., Lohse, M., J., Kobilka, B.K., Caron, M.G. & Lefkowitz, R.J. 1988. The genomic clone G-21 which resembles a β -adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature* 335, 358–360.
- Frederiksson, R. & Schioth, H.B. 2005. The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol Pharm* 67, 1414–1425.
- Gilman, A.G. 1987. G Proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem* 56, 615–649.
- Grinita, L., Shenoy, S.K., Schat, B. *et al.* 2005. β -arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase. *J Biol Chem* 280, 24 412–24 419.
- Hirsch, J.A., Schubert, C., Gurevich, V.V., Sigler, P.B. 1999. A crystal structure of visual arrestin: a model for arrestin's regulation. *Cell* 97, 257–269.
- Holloway, A.C., Qian, H., Pipolo, L. *et al.* 2002. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol Pharmacol* 61, 768–777.
- Hoon, M.A., Adler, E., Lindemeier, J., Battey, J.F., Ryba, N.J. & Zuker, C.S. 1999. Putative mammalian taste receptors: a class of taste-specific GPCRs with distinct topographic selectivity. *Cell* 96, 541–551.
- Kobilka, B.K., Matsui, H., Kobilka, T.S. *et al.* 1987. Cloning, sequencing, and expression of the gene coding for the human platelet α_2 -adrenergic receptor. *Science* 238, 650–656.
- Kobilka, B.K., Kobilka, T.S., Daniel, K., Regan, J.W., Caron, M.G. & Lefkowitz, R.J. 1988. Chimeric α_2 - β_2 adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science* 240, 1310–1316.
- Kubo, T., Fukuda, K., Mikami, A. *et al.* 1986. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* 323, 411–415.
- Kuhn, H. & Wilden, U. 1987. Deactivation of photoactivated rhodopsin by rhodopsin-kinase and arrestin. *J Recept Res* 7, 283–298.
- Lefkowitz, R.J. & Shenoy, S.K. 2005. Transduction of receptor signals by beta-arrestins. *Science* 308, 512–517.
- Limbird, L.E. 1996. In: *Cell Surface Receptors: A Short Course on Theory and Methods*, 2nd edn, pp. 1–26. Kluwer Academic, Boston, MA.
- Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G. & Lefkowitz, R.J. 1990. β -arrestin: a protein that regulates β -adrenergic receptor function. *Science* 248, 1547–1550.
- Lorenz, W., Inglese, J., Palczewski, K., Onorato, J.J., Caron, M.G. & Lefkowitz, R.J. 1991. The receptor kinase family: primary structure of rhodopsin kinase reveals similarities to the beta-adrenergic receptor kinase. *Proc Natl Acad Sci USA* 88, 8715–8719.
- Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. & Nakanishi, S. 1987. cDNA cloning of bovine substance-K receptor through oocyte expression system. *Nature* 329, 836–838.
- Mukherjee, C., Caron, M.G., Coverstone, M. & Lefkowitz, R.J. 1975. Identification of adenylate cyclase-coupled beta-adrenergic receptors in frog erythrocytes with (-)-[3H] alprenolol. *J Biol Chem* 250, 4869–4876.

- Ostrowski, J., Kjelsberg, M.A., Caron, M.G. & Lefkowitz, R.J. 1992. Mutagenesis of the beta2-adrenergic receptor: how structure elucidates function. *Annu Rev Pharmacol Toxicol* 32, 167–183.
- Ovchinnikov, Y.A. 1982. Rhodopsin and bacteriorhodopsin: structure-function relationships. *FEBS Lett* 148, 179–191.
- Perry, S.J., Baillie, G.S., Kohout, T.A. *et al.* 2002. Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science* 298, 834–836.
- Pert, C.B. & Snyder, S.H. 1973. Opiate receptor: demonstration in nervous tissue. *Science* 179, 1011–1014.
- Pitcher, J.A., Freedman, N.J. & Lefkowitz, R.J. 1998. G protein-coupled receptor kinases. *Annu Rev Biochem* 67, 653–692.
- Rodbell, M., Birnbaumer, L., Pohl, S.L. & Krans, H.M. 1971. The glucagon-sensitive adenylyl cyclase system in plasma membranes of rat liver. *J Biol Chem* 246, 1877–1882.
- Shenoy, S.K. & Lefkowitz, R.J. 2003. Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J* 375, 503–515.
- Shenoy, S.K., McDonald, P.H., Kohout, T.A. & Lefkowitz, R.J. 2001. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* 294, 1307–1313.
- Shinohara, T., Dietzschold, B., Craft, C.M. *et al.* 1987. Primary and secondary structure of bovine retinal S antigen (48-kDa protein). *Proc Natl Acad Sci USA* 84, 6975–6979.
- Spiegel, A.M. 1998. In: A.M. Spiegel (ed.) *G Proteins, Receptors and Diseases*. pp. 1–22. Humana Press, Totowa, NJ.
- Stadel, J.M., Nambi, P., Lavin, T.N., Heald, S.L., Caron, M.G. & Lefkowitz, R.J. 1982. Catecholamine-induced desensitization of turkey erythrocyte adenylyl cyclase: structural alterations in the β -adrenergic receptor revealed by photo-affinity labelling. *J Biol Chem* 257, 9242–9245.
- Stadel, J.M., Nambi, P., Shorr, R.G.L., Sawyer, D.F., Caron, M.G. & Lefkowitz, R.J. 1983. Catecholamine-induced desensitization of turkey erythrocyte adenylyl cyclase is associated with phosphorylation of the β -adrenergic receptor. *Proc Natl Acad Sci U S A* 80, 3173–3177.
- Sun, Y., Cheng, Z., Ma, L. & Pei, G. 2002. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem* 277, 49 212–49 219.
- Sutherland, E. 1967. Adenyl cyclase as an adrenergic receptor. *Ann N Y Acad Sci* 139, 703–723.
- Wei, H., Ahn, S., Shenoy, S.K. *et al.* 2003. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* 100, 10 782–10 787.
- Wilden, U. & Kuhn, H. 1982. Light-dependent phosphorylation of rhodopsin: number of phosphorylation sites. *Biochem* 21, 3014–3022.
- Yamamura, H.I. & Snyder, S.H. 1974. Muscarinic cholinergic binding in rat brain. *Proc Natl Acad Sci* 71, 1725–1729.
- Zhao, G.Q., Zhang, Y., Hoon, M.A. *et al.* 2003. The receptors for mammalian sweet and umami taste. *Cell* 115, 255–256.