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G Protein-Coupled Receptors: A Century of Research and Discovery

Samuel Liu[1,*], **Preston J. Anderson**[2,3,*], **Sudarshan Rajagopal**[1,2,4], **Robert J. Lefkowitz**[1,4,5], **Howard A. Rockman**[1,2]

[1]Department of Medicine, Duke University Medical Center

[2]Cell and Molecular Biology (CMB), Duke University, Durham, NC, 27710, USA

[3]Duke Medical Scientist Training Program, Duke University, Durham, NC, 27710, USA

[4]Deparment of Biochemistry Duke University, Durham, NC, 27710, USA

[5]Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710, USA.

Abstract

G protein-coupled receptors (GPCRs), also known as 7 transmembrane domain receptors, are the largest receptor family in the human genome, with approximately 800 members. GPCRs regulate nearly every aspect of human physiology and disease, thus serving as important drug targets in cardiovascular disease. Sharing a conserved structure comprised of seven transmembrane α-helices, GPCRs couple to heterotrimeric G-proteins, GPCR kinases and β-arrestins, promoting downstream signaling through second messengers and other intracellular signaling pathways. GPCR drug development has led to important cardiovascular therapies, such as antagonists of β-adrenergic and angiotensin II receptors for heart failure and hypertension, and agonists of the glucagon-like peptide-1 receptor for reducing adverse cardiovascular events and other emerging indications. There continues to be a major interest in GPCR drug development in cardiovascular and cardiometabolic disease, driven by advances in GPCR mechanistic studies and structure-based drug design. This review recounts the rich history of GPCR research, including the current state of clinically used GPCR drugs, and highlights newly discovered aspects of GPCR biology and promising directions for future investigation. As additional mechanisms for regulating GPCR signaling are uncovered, new strategies for targeting these ubiquitous receptors hold tremendous promise for the field of cardiovascular medicine.

Correspondence: Howard A. Rockman: h.rockman@duke.edu, Or, Robert J. Lefkowitz: lefko001@receptor-biol.duke.edu.
*Contributed equally

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Early history of receptor biology

The tantalizing idea that diseases could be treated with specific chemical substances dates to ancient times and traditions across multiple continents and cultures, but for much of history, the exact mechanism by which these substances acted remained a mystery. In the present day, we now know that one large family of over 800 receptors and their transducers, G protein-coupled receptors (GPCRs), make up the targets of ~30% of all FDA-approved drugs.[1,2] The discoveries surrounding the family of GPCRs are the culmination of pioneering work from countless scientists throughout the 20th century into the modern day, which have resulted in multiple, paradigm-defining discoveries, and Nobel Prizes and which continue to serve as the foundations for innovative fields of biology research today (Figure 1 and 2).

Among the hundreds of clinically targeted GPCRs, the discoveries surrounding the adrenoreceptors, receptors fundamental to normal cardiovascular physiology, make them an excellent case study. The use of naturally existing adrenergic receptor ligands, such as the alkaloid ephedrine from the herb *Ephedra*, can be traced back to ancient Asia.[3] Epinephrine itself, one of the first human hormones to be isolated, was first isolated as a pure crystal from adrenal glands in 1901[4] and initially marketed as a wonder drug.[5,6] Though such naturally occurring adrenergic ligands would see widespread use for centuries, an understanding of their mechanism of action, and of the actual adrenergic receptors they acted on, would only come much later.

Some of the first inquiries in the study of receptors were made by two scientists at the start of the 1900s, Paul Ehrlich and John Langley. Over the course of studying immune responses to pathogens and toxins, Ehrlich would develop a theory of “side-chains,” structures on the surface of cells capable of binding to certain toxins.[7,8] Just a few years later the earliest assertion of receptor function was made by John. N. Langley, who coined the term “receptive substance” in 1905 while studying the contractions of muscle cells stimulated by nicotine. He describes:

> “So we may suppose that in all cells two constituents at least are to be distinguished. The chief substance which is concerned with the chief function of the cell as contraction and secretion and receptive substances which are acted upon by chemical bodies and in certain cases by nervous stimuli. The receptive substance affects or is capable of affecting the metabolism of the chief substance”[9]

Though the identities of the “receptive substances” were a mystery to Langley, these early ideas led to the initial proposal of the receptor concept. The functions of receptors, Langley had clearly postulated, are: first, to interact with external chemical stimuli; and second, to relay these responses to effectors within the cell, generating physiological changes in response. Despite the later-proven accuracy of this theory, these ideas were initially met with

considerable skepticism, and the existence of receptors would remain highly contentious for decades. For instance, consider the perspectives of just a few scientists in the following decades.

In 1943, Sir Henry Dale, who received the Nobel Prize in 1936 for studies on adrenergic and cholinergic neurotransmission and himself a former student of John Langley would say:

> "It is a mere statement of fact to say that the action of adrenaline picks out certain such effector cells and leaves others unaffected; it is a simple deduction that the affected cells have a special affinity of some kind for adrenaline, but I doubt whether the attribution to such cells of "adrenaline receptors" does more than restate this deduction in another form…."[10]

In 1973, Raymond Ahlquist, a distinguished pharmacologist, and recipient of the Lasker prize for his work on the pharmacological differentiation of α and β adrenoreceptor subtypes would remark:

> "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."[11]

The 1955 edition of The Pharmacological Basis of Therapeutics, a standard textbook of pharmacology, states: *"Years ago, Langley named the differentiating substance the 'receptive substance'; this term is still widely employed, but it must be realized that the 'receptor' may not be a morphologically demonstrable structure."*[12] Taken together, these quotes illustrate the dogma in the field at the time.

Despite these limitations to the basic understanding of receptors, an understanding of the physicochemical basis of ligand binding to receptors began to emerge after Langley's initial work, leading to the development of "receptor theory."[13,14] Through the work of Hill (who worked with Langley),[15] Clark,[16] and others, physicochemical models for understanding ligand binding to receptors emerged by developing quantitative relationships between drug action and changes in physiology. Work through the 1950s led by Gaddum,[17] Schild,[18] Ariens,[19] and Stephenson[20] led to the important concepts of affinity, reflecting how tightly a drug bound to its receptor, and efficacy, how effectively the drug:receptor complex promoted a physiological response. But despite these advances, the molecular mechanisms underlying this drug action largely remained a black box.[13]

Insights into the mechanisms of drug action started to change with the discovery of the intracellular signaling pathways regulated by receptors. Research from Earl Sutherland Jr.'s lab first showed that the activity of β-adrenoreceptors resulted in the production of a "second messenger", cyclic adenosine monophosphate (cAMP), which was later shown to be produced by adenylyl cyclase (AC).[21–23] Sutherland's work on AC would be further built upon by the work of Martin Rodbell's lab which determined that AC activation by hormones required the presence of guanosine triphosphate (GTP), presumably through the association of an intermediary protein that bound GTP.[24–26] These discoveries would be further built on by Alfred Gilman's lab, which successfully identified and isolated the intermediary

heterotrimeric G-protein.[27,28] For this work, Sutherland received the Nobel Prize in 1971 and Rodbell and Gilman shared the Nobel Prize in 1994.

β-adrenergic receptors as the prototype for GPCRs

Among the many GPCRs, β-adrenergic receptors were used as the model system in many early studies to understand GPCR structure and function. The importance of β-adrenergic signaling in cardiovascular physiology led to the development of antagonists of the β-adrenergic receptor, commonly referred to as "beta-blockers." With the hypothesis that inhibiting adrenergic signaling would diminish the heart's demand for oxygen in the setting of angina, Sir James Black led the development of the first beta-blocker in the 1960s, at a time without knowledge of receptor structure or how adrenergic ligands bound to the receptor, and when the existence of receptors themselves was still questionable. Black ultimately identified the first clinically utilized beta-blocker, propranolol, synthesizing and screening numerous derivatives of catecholamines, applying mathematical models of affinity and efficacy to identify competitive antagonists for the receptor.[29,30]

The development of the first small-molecule antagonists paved the way for the use of ligands as tools to label, capture, and purify adrenergic receptors themselves, work which was pioneered in the Lefkowitz laboratory. Radiolabeling β-adrenergic-specific agonists and antagonists provided a highly specific method for detecting receptors.[31–33] The binding of agonists to receptors was found to be biphasic, showing two distinct affinity states (high and low) due to the allosteric effects of G-proteins on the receptors. Notably, the addition of guanine nucleotides converted high to low-affinity state receptors (by promoting heterotrimeric G-protein dissociation).[34] This ultimately led to the development of the ternary complex model to explain the allostery between receptors, agonists, and heterotrimeric G-proteins.[35] Concurrently, work on chemical conjugation of the beta-blocker, alprenolol, onto gel columns allowed for the development of affinity chromatography techniques to capture and purify the β2-adrenergic receptor (β_2AR), one of the three β-adrenergic receptors expressed in the heart.[36] This in turn ultimately facilitated the cloning of the β_2AR gene.[37] Purified receptors were chemically cleaved into short peptides, several of whose amino acid sequences were determined, thus permitting the design of oligonucleotide probes which were used to clone the receptor gene and cDNA.[37] The cloning of the β_2AR was a watershed event, since it revealed the similarity of its primary structure and seven transmembrane domain architecture to the retinal "light receptor" rhodopsin. In the years after, several additional GPCRs, including the β_1AR, the predominant β-adrenergic receptor expressed in the heart, would also be cloned using a similar strategy. Subsequently, hundreds of receptors would be shown to share these features and these insights marked the discovery of the superfamily of seven transmembrane receptors that regulate virtually all of human physiology.

Rapid progress in understanding the mechanisms regulating GPCR signaling was made in the Lefkowitz lab during the 1980s and 1990s. After heterotrimeric G-proteins are activated by GPCRs, the receptors rapidly "desensitize," preventing uncontrolled signaling acutely, and then in the face of prolonged signaling "downregulate," due to their destruction in lysosomes with decreased receptor expression to decrease further signaling and maintain

homeostasis. This process was shown to require receptor phosphorylation and through analogy with rhodopsin, kinases that we now refer to as GPCR kinases (GRKs).[38] Desensitization further requires the activity of adapter proteins known as β-arrestin-1[39] and 2[40], which share homology with a retinal protein originally known as S-antigen (now known as visual arrestin) which had been shown to quench rhodopsin signaling.[41] This family of 4 proteins consists of arrestins 1 and 4 with expression restricted to the retina, and β-arrestin-1[39] and 2[40] (aka arrestins 3 and 4) which are expressed ubiquitously. β-arrestins were also shown to promote endocytosis and downregulation of most receptors.[42,43] Despite their initial discovery of "arresters" of signaling, β-arrestins were later shown to also promote signaling through kinase cascades[44] and other mechanisms, displaying their multifunctional nature in the regulation of receptor desensitization, trafficking, and signaling.

Of the 3 βARs subtypes (β1AR, β2AR, and β3AR) expressed in the heart, β1ARs are the most abundant (~75–80%) with some expression of β2ARs (~15–20%) and minimal expression of β3AR.[45,46] Subsequent discoveries led to additional understanding of their roles in the pathophysiology of heart disease, such as the observations of beta-receptor downregulation[45,47] and altered expression of GRKs[48] in heart failure. Studies investigating levels of beta receptor ligands found them useful as biomarkers for HF progression, ultimately culminating in the landmark paper in 1984 proposing the neurohormonal hypothesis of HF: the idea that adverse cardiac remodeling and progression of heart failure is dependent on overactivation of the autonomic sympathetic nervous system.[49] Subsequent work in experimental systems found that transgenic mice with elevated β_1AR signaling, either through β_1AR overexpression[50] or $G\alpha_s$ overexpression,[51] spontaneously develop heart failure while transgenic mice that overexpress β_2AR in the heart display enhanced cardiac function.[52]

The increasing understanding of βAR dysfunction in heart failure came at a time when there was a long-standing belief that beta-blockers were contraindicated in the setting of heart failure due to their negative inotropic properties. Over time, these views became increasingly challenged, and several major trials in the 1980s and 1990s conclusively demonstrated that beta-blockers substantially improved survival,[53–57] although this effect was only observed in beta-blockers that did not have weak partial agonist activity, known as "intrinsic sympathomimetic activity".[58–60] Beta-blockers would prove to be immensely successful in the treatment of heart failure, and the story of these adrenoreceptor ligands illustrates the rich history of this family of receptors, the dramatic advances in their understanding over the past century, and the immense therapeutic potential that they hold.

Molecular machinery underlying GPCR signaling

GPCRs share a conserved 7 transmembrane domain structure with an extracellular-facing ligand binding site and an intracellular pocket for transducer binding. GPCRs also have 3 intracellular loops and a C-terminal tail which regulate transducer interactions. GPCRs are activated by ligand binding to the GPCR, inducing conformational changes that allow for the subsequent recruitment of effectors. GPCRs signal through a variety of effectors, most prominently heterotrimeric G-proteins, GRKs, and β-arrestins (Figure 3).

Heterotrimeric G proteins

Heterotrimeric G-proteins, the largest family of GPCR signaling transducers, are composed of a complex of Gα, G_{β}, and G_{γ} subunits. Upon receptor activation, the receptor acts as a guanine nucleotide exchange factor for the Gα subunit, inducing release of guanosine diphosphate (GDP) in exchange for guanosine triphosphate (GTP). This induces dissociation of the Gα-GTP subunit from the $G_{\beta\gamma}$ complex, allowing each of these units to independently interact with a wide range of effectors to regulate second messenger levels, protein kinases, and other pathways to impact different cellular functions.[61]

There are sixteen Gα subunits that fall into four main families: $G\alpha_{s}$, $G\alpha_{i}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, all of which differentially engage with a variety of effectors. Different receptors preferentially activate specific Gα subunits allowing for highly specific signaling pathways to occur downstream of receptor activation. $G\alpha_{s}$ subunits stimulate AC to convert adenosine triphosphate (ATP) to cAMP and lead to the activation of protein kinase A (PKA)[62] and other targets. In the heart, PKA phosphorylates a wide array of effectors such as troponin I, myosin-binding protein-C, phospholamban, the cardiac ryanodine receptor (RYR2), and voltage-gated L-type Ca^{2+} channels.[63] βAR stimulation collectively orchestrates these subcellular components to enhance myofilament cross-bridge cycling to increase the force of contraction (inotropy) and the rate of relaxation (lusitropy). Action potential activated L-type Ca^{2+} channels initiate inward Ca^{2+} entry and trigger a large release of intracellular Ca^{2+} by the RYR2 through a process known as Ca^{2+}-induced Ca^{2+} release. This triggered Ca^{2+} release by the RYR2 was defined as a Ca^{2+} spark[64] and is the primary source of intracellular Ca^{2+} available for myofilament cross-bridge formation and contraction. βAR stimulation also regulates heart rate (chronotropy) via the Hyperpolarization-activated Cyclic Nucleotide-gated channel and other ion channels and transporters in the sinoatrial node.[65] In contrast, receptors that couple through $G\alpha_{i}$ inhibit AC, therefore reducing cAMP production and its downstream effects on the heart.

$G\alpha_{q/11}$ activates phospholipase-Cβ to convert membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 activates cardiomyocyte IP_3 receptors, which are intracellular Ca^{2+} release channels embedded in the sarcoplasmic reticulum (SR) and nuclear envelope. GPCR stimulated IP_3 production elicits local nuclear envelope Ca^{2+} release via IP_3 receptors to activate cardiac hypertrophic signaling, in part, through the Ca2+ and calmodulin-dependent serine/threonine protein phosphatase calcineurin[66] and a nuclear pool of Ca^{2+}-calmodulin–dependent protein kinase II.[67] IP_3 stimulated release of this local Ca^{2+} pool is distinct from the cytoplasmic Ca^{2+} transient involved in excitation-contraction coupling[66,67]. DAG and intracellular Ca^{2+} activate protein kinase C-alpha (PKC-a) which regulates cardiac contractility through upregulation of type 1 protein phosphatase leading to dephosphorylation of the SR Ca^{2+}ATPase pump regulating protein phospholamban.[68]

$G\alpha_{12/13}$ are known to activate the small GTPase RhoA,[69,70] which can activate downstream kinases, regulating vascular smooth muscle tone.[71–73] Beyond these canonical signaling pathways, each of these Gα subunit families can regulate a wide array of relatively poorly characterized signaling pathways. Furthermore, in addition to the Gα subunits, there are 5

Gβ subunits and 12 Gγ subunits, which also regulate a wide range of signaling pathways with distinct spatial and temporal profiles.[74,75]

Heterotrimeric G protein signaling is essential for normal cardiac function but chronic sustained stimulation, particularly of $G\alpha_s$ and $G\alpha_{q/11}$, can lead to deleterious adverse ventricular remodeling and depressed cardiac function. In the setting of the heart, the negative consequences of overactive G-protein signaling include tachycardia and progression of heart failure.[76] In contrast, activation of $G\alpha_i$ can play an inhibitory role on AC activity and may be beneficial for opposing $G\alpha_s$ signaling. Thus, negative feedback loops are crucial in maintaining cellular homeostasis after GPCR stimulation. Active Gα-GTP is turned off by regulators of G protein signaling (RGS) proteins, which act as GTPase activating proteins (GAPs) that promote hydrolysis of GTP to Gα-GDP.[77] This, in turn, leads to the termination of Gα-protein-mediated signaling and reassociation of the heterotrimeric G-protein complex. Complex mechanisms promote the process of receptor desensitization, in which the ability of active receptors to signal is diminished, otherwise signaling would proceed unabated.

GRKs and Other Kinases

Desensitization is promoted through receptor phosphorylation by kinases that phosphorylate serine/ threonine motifs in the receptor's intracellular loops and C-tail. These phosphorylation patterns either directly interfere with the receptor's normal signaling functions or lead to the recruitment of β-arrestins that sterically interfere with G-protein coupling and induce receptor internalization.

There are two types of processes by which kinases regulate receptor desensitization: homologous desensitization, in which kinases are recruited to and phosphorylate an agonist-activated receptor, or heterologous desensitization, in which kinases phosphorylate specific motifs on receptors regardless of their activation state. Homologous desensitization is promoted primarily by GRKs, which are recruited through an interaction with the core of the active receptor, and which phosphorylate the receptor intracellular loops and C-terminal tail.[78,79] Heterologous desensitization is mediated primarily by second messenger-dependent kinases such as PKA and PKC, which recognize specific motifs in receptor intracellular domains. Thus, even in the absence of its cognate ligand, a receptor can undergo heterologous desensitization. For example, cAMP or cAMP analogs promote phosphorylation of the β_2AR through the activity of PKA, thereby reducing the receptor's ability to signal through $G\alpha_s$[80]. A related phenomenon is class switching, where phosphorylation changes the coupling specificity of a receptor. Examples include the β_2AR, where PKA phosphorylation leads to the receptor changing its preferential coupling from $G\alpha_s$ to $G\alpha_i$,[81] further inhibiting signaling through AC. Similarly, for the glucagon-like peptide-1 receptor (GLP-1R), PKC modulates a signaling switch from $G\alpha_s$ to $G\alpha_q$.[82]

Regulation of receptor phosphorylation is highly dependent on cellular context. There are 7 GRKs in humans, all of which share a common tripartite structure: a GPCR binding domain, a kinase domain, and a regulatory domain.[83] Based on structural and sequence homology, they are further subdivided into three subfamilies: the GRK1 family(GRK1 and 7); the GRK2 family (GRK2 and 3); and the GRK4 family (GRK4, 5, and 6). GRK1

and 7 are primarily found in retinal rod and cone cells respectively.[84] GRK2 and 3 both contain a C-terminal pleckstrin homology (PH) domain, which promotes their localization to the plasma membrane via Gβγ binding.[85] GRKs 4 and 6 carry palmitoylation sites, and GRK 5 contains a positively charged lipid binding element. As a result, GRK 4, 5, and 6 do not require interaction with $G_{\beta\gamma}$ to localize to the plasma membrane. Adding to the context-dependent activity of these kinases, different GRKs and PKA/PKC also induce different phosphorylation patterns at the receptor, with distinct impacts on receptor signaling and recruitment of other proteins to the receptor.[86–88] Taken together, the function of these protein kinases is to fine-tune GPCR signaling responses.

In addition to the GPCRs a number of nonreceptor substrates have been shown to be phosphorylated by or interact with these kinases such as receptor tyrosine kinases, cytoskeletal proteins,[89] and phosphoinositide 3-kinase (PI3K).[90,91] PKA and GRK5 can also serve as adapter proteins and translocate to the nucleus,[92–95] where they can regulate gene expression.

β-arrestins: Canonical GPCR transducers

Arrestins are a family of four multifunctional adaptor proteins with three main functions at the receptor: desensitization, trafficking, and signaling. There are four arrestins that share high sequence and structural homology, containing an N-terminal domain, a central core domain, and a C-terminal tail. Arrestin 1 (aka visual arrestin) and 4 (aka x-arrestin) are found exclusively in rod and cone cells while β-arrestins 1 and 2 (aka arrestins 2 and 3) are found ubiquitously.[96] While β-arrestins 1 and 2 share many structural and functional similarities, they also have their own distinct cellular functions. For example, β-arrestin 1 and −2 both have nuclear localization sequences on the N-terminus, but β-arrestin 2 has a nuclear export sequence on the C-terminus resulting in differential nucleocytoplasmic shuttling of the β-arrestins.[97]

Upon receptor phosphorylation, β-arrestins are recruited to the receptor through an interaction with the receptor's phosphorylated tail. Additional interactions with the intracellular loops and core of activated receptors permit a range of conformational states of β-arrestin:receptor complexes.[98,99] This recruitment of β-arrestins sterically interferes with receptor interactions with the Gα subunit, desensitizing the receptor by restricting further signaling by G-proteins.

Simultaneously, binding of β-arrestin to a receptor induces conformational changes in β-arrestin, which allow it to interact with hundreds of proteins involved in a wide range of functions.[100,101] β-arrestin then promotes the process of receptor internalization, decreasing receptor expression at the plasma membrane by serving as an adaptor for the endocytotic machinery through AP2 and clathrin binding motifs found on its C-tail.[43,102,103] This promotes the translocation of the receptor to intracellular compartments for recycling or degradation.[79] Receptor:β-arrestin interactions play an important role in specifying the specific targeting of receptors to intracellular compartments. For example, receptors that have high-affinity for β-arrestin, such as the vasopressin 2 receptor (V_2R) and angiotensin II receptor type 1 (AT_1R), experience sustained internalization, often proceeding to lysosomes

for degradation. In contrast, receptors such as the β_2AR, which have a relatively low affinity for β-arrestin, are recycled rapidly back to the cell surface after internalization.[102]

Receptor-activated β-arrestin also serves as a scaffold and allosteric activator for protein kinase signaling cascades, amplifying the activity of these pathways within cells. These include mitogen-activated protein kinases (MAPK), PI3K, AKT, and Src[44,90,104–107] in addition to a wide range of other pathways.[108] G-protein and β-arrestin-mediated signaling display different spatial and temporal profiles,[109] although this can vary significantly among receptors. Even when activating the same distal effector, they often mediate different cellular sequelae due to differential subcellular compartmentalization of the activated effectors. For example, the AT_1R, an important receptor with key roles in blood pressure regulation, vascular function, and cell remodeling signals to ERK through both G protein and β-arrestin transducers. In response to stimulation by the endogenous ligand, angiotensin II (AngII), AT_1R $G\alpha_q$-activated ERK translocates to the nucleus, where it activates transcriptional networks. In contrast, β-arrestin sequesters ERK in the cytosol resulting in the phosphorylation of cytosolic proteins resulting in activation of different cellular responses.[109] In the heart, signaling via β_1AR-mediated Epidermal Growth Factor Receptor (EGFR) transactivation is cardioprotective against chronic catecholamine stimulation[110] through a β-arrestin dependent mechanism mediated, in part, through differential intracellular trafficking of ERK1/2.[111] At the molecular level, there appears to be functional specialization between β-arrestin isoforms. At the AT_1R, recruitment of β-arrestin 1 and 2 are similar, but siRNA knockdown of β-arrestin 1 results in increased ERK signaling, whereas knockdown of β-arrestin 2 results in decreased ERK signaling. This suggests that β-arrestin 2, but not β-arrestin 1, mediates ERK1/2 activation at the AT_1R.[112,113] On the other hand, at the β_2AR, knockdown of either β-arrestin 1 or 2 results in diminished ERK signaling, suggesting that β-arrestins mediate different responses at different receptors.[106] While β-arrestin-mediated responses are thought to be distinct from those mediated by G-protein, more recently, it has also been appreciated that β-arrestins can promote G-protein signaling from internalized GPCRs.[114,115] While β-arrestins were originally identified for their role in desensitizing G protein signaling by GPCRs at the plasma membrane, they can actually promote signaling from internalized receptors. These contrasting functions further highlight the diversity of cellular responses mediated by β-arrestins.

Other GPCR-binding Proteins

Individual GPCRs are also capable of interacting with a wide range of proteins depending on the presence of specific motifs in their intracellular domains.[116] These GPCR-interacting partners can sometimes directly mediate receptor signaling or act as scaffolds to modulate signaling. These include the AKAPs, Homer, JAK2, and NHERF1 (reviewed in[116]).
One important family of GPCR-interacting proteins is the receptor activity modifying proteins (RAMPs).[117] The activity of RAMPs greatly modifies the behavior of GPCRs. For the calcitonin-like receptor (CLR), association with RAMP1 results in a receptor for the calcitonin gene-related peptide (CGRP), promoting vasodilation in diseases such as migraine.[118] This is in contrast to the properties of CLR in association with RAMP2, which generates an adrenomedullin 1 receptor where adrenomedullin has the highest potency for

vasodilation, and association with RAMP3, which generates an adrenomedullin 2 receptor where adrenomedullin 2 and adrenomedullin have similar potency.[117] Originally thought to only interact with a limited number of family B receptors, recent studies have now demonstrated that RAMPs interact with a much wider range of GPCRs.[119,120] This likely is the tip of the iceberg, as other receptor-interacting proteins likely play additional roles in GPCR biology.

Biased Agonism

As GPCRs can signal through multiple pathways promoted by different G proteins, GRKs, β-arrestins, and other interacting proteins, it has been appreciated that in different contexts, the same receptor can promote distinct patterns of signaling outcomes through selectively activating subsets of transducers (Figure 4).[121] The ability of a GPCR to selectively or preferentially couple to distinct transducers is now referred to as *biased agonism*,[121] and can be induced through a variety of mechanisms. The first discovered form of bias was *ligand bias,* referring to the phenomenon whereby different ligands for a GPCR were found to have different efficacies for activating downstream transducers, such as preferentially recruiting β-arrestins, leading to a biased response compared to the reference endogenous agonist.[122] Ligand bias is thought to be due to the ability of different agonists to promote distinct conformational states of the receptor that have different efficacies for engaging with different transducers and initiating signaling through different signaling pathways.[123] It is now appreciated that all agonists lie on a spectrum of bias through their stabilization of different receptor conformations.[122]

There are additional mechanisms that may underlie a biased cellular response, such as *receptor*, *system,* and *location* bias. *Receptor* bias (Figure 4) refers to bias at the level of the receptor itself, where a receptor inherently couples more effectively to one transducer or another compared to a reference. The existence of such receptors can be demonstrated by experimentally generated receptors where important residues for receptor activation and transducer coupling have been mutated such as the AT_1R “AAY” or β_2AR “TYY” mutant receptors which show biased coupling preferences compared to their unmodified wild-type receptors.[106,112] Additionally, naturally existing receptor variants that lead to biased signaling profiles have also been identified in a number of disease states.[124] *System bias* (Figure 4), refers to the differential expression of individual signaling components, such as G proteins, β-arrestins, and GRKs, which can lead to tissue/cell type-specific differences in signaling by the same agonist:receptor complex.[121] Such situations can occur in disease states where there is differential expression of receptors, transducers, and effectors.[125,126] A related concept is that of *location bias* (Figure 4), where different ligands promote receptor activation in different subcellular locations, resulting in distinct cellular and physiological effects.[127]

As bias allows for the selection of signaling pathways to be activated downstream of a receptor, biased ligands have the potential to activate beneficial signaling pathways while limiting off-target effects at a given GPCR. Thus, drug development of biased agonists is a very active area of research. For example, activation of the AT_1R by its endogenous ligand AngII in rats initiates signaling through both G-protein and β-arrestin dependent

pathways, enhancing cardiac contractility while inducing cardiac hypertrophy. In constrast the synthetic, β-arrestin biased AT_1R agonist TRV027 enhances contractility but does not induce cardiac hypertrophy.[128] Bias is also highly pertinent to the elucidation of GPCR function in their endogenous context, particularly location bias and system bias. For example in cardiomyocytes, numerous receptors are expressed, such as βAR, α1AR, and AT_1R, in various cellular compartments and the nucleus, where this spatial localization contributes to their unique signaling functions.[129–131] These topics will be further explored in the **Emerging Directions** section at the end of the review.

Important GPCR Targets in Cardiovascular Medicine

There are more than 200 GPCRs expressed in the heart,[132] and drugs targeting many of these GPCRs expressed in the cardiovascular system are mainstays of clinical treatment for a wide range of pathologies. Here we highlight three families of GPCRs with historical, clinical, and emerging importance in cardiovascular medicine: βARs, AT_1Rs, and incretin receptors (Table 1).

β-Adrenergic Receptors

The β_1AR and β_2AR are the predominant adrenergic receptor subtypes expressed in the heart, while the β_3AR is primarily expressed in adipose tissue. In the normal heart, the β_1:β_2 ratio is 80:20,[45] while in heart failure, the β_1:β_2 ratio is reduced to 60:40, due to the downregulation of the β_1AR.[47] In cardiac myocytes, β_1ARs predominate with β_2 and β_3ARs mostly found in nonmyocytes.[46] Upon stimulation with its endogenous ligands epinephrine and norepinephrine, the β_1AR couples to $G\alpha_{s,}$ the β_2AR to $G\alpha_s$ and $G\alpha_i$, and the β_3AR to $G\alpha_s$. Activation of βARs generally initiates a Gs-AC-cAMP-PKA signaling cascade, increasing myocardial contraction and heart rate. However, long-term activation of $G\alpha_s$ signaling by βARs in heart failure can lead to pathological remodeling of heart tissue and their downregulation.[133]

As noted earlier, beta-blockers are one of the most widely used therapeutics for numerous diseases including hypertension, coronary artery disease, heart failure, and arrhythmias.[54,134] However, initially, the use of beta-blockers for heart failure was seen as a contraindication, and it took nearly 30 years from the discovery of beta-blockers to the first clinical trials for use in heart failure. In the 1990's, some of the earliest beta-blocker clinical trials of metoprolol were associated with lower mortality rates[135] (Table 1). This reinvigorated interest in beta-blockers, and a third-generation beta-blocker, carvedilol, was shown to reduce cardiovascular-related hospitalization and death.[54] Although carvedilol is an FDA-approved beta-blocker, the complexities of its pharmacology are still being uncovered. At the β_2AR, carvedilol is a β-arrestin-biased agonist with inverse agonism for $G\alpha_s$ signaling.[136] At the β_1AR, carvedilol displays β-arrestin-biased signaling dependent on $G\alpha_i$, regulates microRNA processing, activates ERK signaling,[137–139] and provides cardioprotection in response to ischemia-reperfusion.[140] β_1AR mediated β-arrestin transactivation of the epithelial growth factor receptor (EGFR), requires β-arrestin, GRK5 and/or 6.[110] Activation of this transactivation pathway is cardioprotective against catecholamine toxicity.[110] Though it has been reported that carvedilol can activate

$G\alpha_s$,[141] such findings have not been observed in most physiologically-relevant systems and run counter to numerous clinical studies showing that beta-blockers with intrinsic sympathomimetic activity are associated with negative outcomes in heart failure.[58–60] Furthermore, carvedilol also has additional α1 adrenergic blockade and antioxidant properties, which have all been theorized to contribute to its unique cardioprotective properties.[142,143] At the moment, the question of what specific properties will result in the development of "better" beta-blockers is still under investigation, though research in our laboratories has focused on evaluating and enhancing the effects of β-arrestin-biased signaling of beta-blockers.[140,144]

The Type 1 Angiotensin II Receptor

The renin-angiotensin-aldosterone system (RAAS) plays a central role in regulating blood pressure and volume status through its effects on the cardiovascular system and kidneys.[145] Renin cleaves angiotensinogen into angiotensin I, which is further processed by angiotensin-converting enzyme in the lungs into AngII. The angiotensin II receptor type 1 (AT_1R) and type 2 (AT_2R) appear to have contrasting physiological roles. In the cardiovascular system, the AT_1R promotes vasoconstriction, and vascular smooth muscle cell proliferation, while AT_2Rs promote vasodilatation and inhibit proliferation. In heart failure, AT_1R mRNA expression is downregulated, whereas there is no change for AT_2R.[146] AT_1R signaling also promotes the secretion of aldosterone by the adrenal cortex and promotes sodium retention in the kidney.

AT_1Rs can signal through a wide range of pathways.[147–150] Upon activation by its endogenous ligand, AngII, the AT_1R signals through $G\alpha_q$, but can also couple to other G proteins, such as $G\alpha_{12/13}$ and $G\alpha_{i/o}$. In addition, the AT_1R can signal via β-arrestins to multiple effectors, such as MAP kinases. Furthermore, the AT_1R is also capable of promoting signaling through other transmembrane receptors, such as the EGFR through β-arrestin-dependent and -independent mechanisms.[151–153]

FDA-approved RAAS inhibitors include direct renin inhibitors, angiotensin-converting enzyme inhibitors (ACEIs), AT_1R antagonists (ARBs), and mineralocorticoid receptor antagonists. ACEIs and ARBs are central to the treatment of hypertension, heart failure with reduced ejection fraction, and chronic kidney disease.[154,155] One advantage of ARBs over ACEIs is the lower risk of side effects such as cough and angioedema, the latter of which can be life-threatening due to swelling and obstruction of the airway.[156] In 1987, there was great optimism surrounding ACEIs for their use in congestive heart failure following the CONSENSUS trial.[157] (Table 1). This study showed that adding enalapril to a conventional heart failure regimen that at the time was typically furosemide and digoxin, reduced mortality by 40% at 6 months and 27% compared to the end of the 12-month study. Following the positive studies and enthusiasm for ACEIs, there was interest that ARBs could block RAAS more effectively compared to ACEIs. In 1997 the ELITE trial, compared the ARB losartan, with the ACEI captopril. There was a 32% reduction in death and hospital admissions for heart failure treated with losartan compared with those treated with captopril.[158] In addition, losartan was better tolerated compared to captopril, with fewer patients discontinuing therapy. More recently, the combination of the ARB valsartan with a

neprilysin inhibitor (sacubitril) has demonstrated superiority to enalapril in the treatment of heart failure with reduced ejection fraction in the PARADIGM-HF trial.[159]

Although ACEIs and ARBs are currently mainstays in the treatment of hypertension and heart failure, AT_1R β-arrestin biased agonists have a theoretical advantage over ARBs that block both G proteins and β-arrestins.[160,161] $G\alpha_q$ appears to mediate the majority of pathological actions of chronic AT_1R activation in the setting of heart failure and is the primary mechanism that drives the hypertrophic response to pressure overload.[162] Studies in animal models have documented the positive effects of AT_1R β-arrestin-biased agonists including improved cardiac output, hemodynamic profile, and preserved renal function while retaining the antihypertensive effects of traditional angiotensin receptor blockers.[163,164] β-arrestin biased AT_1R signaling has been shown to increase isolated myocyte contractility and in vivo cardiac contractility,[160,165] and be necessary for the Frank-Starling law of the heart.[166] There has been a single clinical trial of the β-arrestin biased ligand TRV0027 in the setting of acute systolic heart failure, where the drug failed to show evidence of a beneficial effect.[167] However, a range of factors, including the study design and the short duration of treatment could have explained the observed lack of efficacy in acute HF. Additionally, a later post-hoc analysis found TRV027 treatment was associated with reduced all-cause and cardiovascular death[168]. Therefore, the question as to whether β-arrestin biased AT_1R agonists will live up to their therapeutic promise for chronic HF remains open.[169]

Incretin hormone receptors

Glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP), are two peptide hormones released by the gut that are responsible for mediating increased insulin secretion by the pancreas in response to an oral glucose load. Their receptors are expressed on the beta cells in pancreatic islets and are also expressed in the heart, vasculature, intestines, kidney, and brain.[170,171] The GLP-1 receptor (GLP-1R) is $G\alpha_s$- and $G\alpha_q$-coupled while the GIP receptor (GIPR) only signals through $G\alpha_s$. The relative ability of both receptors to signal through $G\alpha_s$ may be altered during diabetes.[82] Both receptors can also signal through β-arrestin.[170]

Though GLP-1R agonists were originally developed and approved for glycemic control in the setting of diabetes, incretin receptor agonists have been found to improve a much wider range of health outcomes. A recent meta-analysis focusing on cardiovascular and kidney outcomes in diabetics found that use of GLP-1R agonists improved a number of cardiovascular biomarkers and reduce all-cause major adverse cardiovascular events, a composite outcome including nonfatal myocardial infarction, stroke, and cardiovascular death.[172] Additionally, in the more recent SELECT[173] and STEP-HFpEF[174] clinical trials, the GLP-1R agonist semaglutide improved cardiovascular outcomes in patients with obesity in the absence of diabetes. In the wake of these promising findings, GLP-1 receptor agonists have been recognized as “the breakthrough of the year 2023”.[175]

The mechanism by which GLP-1R agonists promote beneficial cardiovascular outcomes is multifactorial. The treatment of obesity, which increases cardiovascular disease risk through the development of dyslipidemia, type two diabetes (T2D), and hypertension, likely plays

a role.[176,177] Additionally, some of their activities appear to be mediated through direct actions on receptors expressed in the cardiovascular system. For example, GLP-1Rs appear to regulate platelet activation[178,179], vasodilation[180], and inflammation.[181] However, the relative contributions of these mechanisms to cardiovascular outcomes are unclear.[171]

Currently under development and in clinical trials are additional incretin receptor agonists. Here the focus has been on three fronts. First, is the development of small molecule agonists of incretin receptors that have improved oral bioavailability compared to currently existing peptide-based agonists.[182,183] Second, is the development of triple agonists that have the ability to activate GLP, GIP, and glucagon receptors, and which appear to have greater efficacy for glycemic control and weight loss.[184,185] Finally is the investigation of the role biased agonism plays in the physiological response to these ligands[186,187]. Physiologically, insulin secretion in pancreatic beta cells in response to incretin stimulation is mediated by $G\alpha_s$, $G\alpha_q$, and β-arrestin.[82,188,189] Recent structural studies have provided some insight into the mechanisms underlying ligand bias at the GLP-1R.[190] Further research is needed to identify the benefits of biased signaling with respect to the mechanisims of these drugs and their relation to cardiovascular outcomes.

Emerging Paradigms in GPCR Biology and Human Health

The past century has seen dramatic advancements in our understanding of GPCR biology across multiple scales of biological organization. Current and emerging research paradigms include 1) the structural mechanisms underlying GPCR activation and transducer engagement; 2) the use of these data in approaches to structure-based drug discovery; and 3) location/context-dependent mechanisms underlying GPCR signaling. Here we highlight exciting developments in each of these areas and discuss their future directions.

Structural features underlying GPCR activation and transducer engagement

GPCR Structural Biology—The first structure of a GPCR bound to a diffusible ligand to be solved was the β_2AR in 2007 by a group led by Brian Kobilka.[191,192] Four years later, researchers led by the same group achieved an even more substantial accomplishment, a crystal structure of the β_2AR bound to $G\alpha_s$ transducer.[193] This first snapshot of a GPCR bound to a transducer provided tremendous insights into the structural basis of GPCR transmembrane signaling, identifying broad structural rearrangements of both receptor and transducer, and the specific amino acid interactions between the receptor and transducer stabilizing these interactions.

Since the structural determination of the β_2AR in 2007, the number of solved structures of GPCRs available in the protein data bank (PDB) has increased dramatically. Within a year of solving the β_2AR, three additional structures of other GPCRs had been solved. By the end of 2023, the number has increased to 180 unique receptors. In all, of the 180 unique receptors, there are over 1,000 structures of GPCRs in varying conformations occupied by different ligands, or coupled to different transducers.[194,195] Furthermore, computer prediction models such as AlphaFold have the potential to produce models of the over 600 receptors whose structures have not yet been solved experimentally.[196]

Improvements in the structural determination of GPCRs have been driven by several methodological advances. They range from improved detergent systems, stabilizing mutations or fusion proteins, conformation-specific nanobodies, and technical improvements in X-ray crystallography and cryo-EM.[197] For example, methodological advances necessary for solving the structure of the human β_2AR included sufficient expression of the β_2AR through virus-based expression methods and further stabilization by the addition of either a receptor-specific fragment antigen-binding region (Fab) or receptor modifications, such as an engineered T4L lysozyme.[191,192] Advancements in cryo-EM have sparked a further GPCR structural revolution,[197] with an increasing number of receptor:transducer complex structures stabilized by nanobodies or Fabs.

Towards an understanding of GPCR dynamics—Though techniques for obtaining GPCR structures have advanced considerably, a key limitation of these methods is that they can only provide singular snapshots of a GPCR - ones that are highly influenced by the particular crystallization/cryo-EM conditions, and by the artificial presence of stabilizing mutations or antibodies (Figure 5A). Furthermore, as opposed to static structures, receptors are highly dynamic and adopt multiple conformations, with distinct conformations mediating specific signaling outcomes.[121] As a result, the study of these conformations and their dynamics is essential to our understanding of GPCR biology. Therefore, the ability to characterize the structural differences and dynamics of receptor conformations is paramount. Two methods have come to the fore: nuclear magnetic resonance (NMR) spectroscopy and site-directed spin labeling electron paramagnetic resonance (EPR) spectroscopy (Figure 5B–C).[198,199] These methods rely on labeling specific residues within receptors and measuring changes in their chemical environment (NMR) or changes in distances from other labeled residues (EPR). The concept of conformational heterogeneity is especially important in the study of biased agonism, as the presence of multiple conformations explains the ability of receptors to interact with distinct transducers with different efficiencies. For example, NMR studies of the β_2AR occupied by ligands with varying biases have provided evidence of unique conformational states in helix VII associated with β-arrestin-biased states.[200] Similar conformational studies have been performed on the μ opioid and adenosine receptors.[201,202] In a landmark study of a panel of AT_1R-biased agonists, distinct populations of AT_1R conformations stabilized by biased ligands were uncovered with EPR using a specific EPR technique known as Pulsed EPR double electron–electron resonance (DEER) spectroscopy.[123]

New Approaches to GPCR Drug Discovery

Structure-based Drug Design—The wealth of structural information on GPCR has ushered in a “golden” age of structure-based drug design for GPCRs. The increasing availability of high-quality receptor structures combined with advancements in virtual drug screening have played key roles in this process, and have already been applied to design ligands for numerous receptors.[203,204] Facilitating this boon of structure-based drug design have been improvements in computational hardware, refined screening/docking algorithms, and optimizations of virtual libraries for screening. For example, a recent virtual screening campaign at the melanocortin 1 receptor was able to screen more than 150 million compounds; the top 300,000 compounds were further sorted and forty representative

compounds from the top 0.1% were synthesized.[205,206] Surprisingly, of the synthesized compounds, 15 compounds showed activity, with a final hit rate of close to 40% using this screening strategy. Novel approaches such as synthon-based ligand discovery allow the virtual screening of billions of compounds.[207] Despite the success of such screens, there still is room for refinements in pose prediction programs and the ability to evaluate compound binding from the perspective of receptor dynamics.[203,204] Further challenges of structure-based drug discovery are to tackle the potential binding at multiple allosteric sites and screening for biased agonists (Figure 5D).[208]

Allosteric Modulators—Traditional GPCR-targeted drug development has focused primarily on ligands that bind to the receptor's extracellular facing orthosteric ligand binding site. However, receptor activation is linked to conformational changes across the entire receptor, with the existence of multiple conformationally distinct inactive and active states[209] (Figure 6A). As a result, the receptor surface contains additional sites that can be targeted by *allosteric* modulators. These allosteric modulators often bind nearby key activation switches on the receptor surface such as the conserved aspartic acid-arginine-tyrosine (DRY) motif, and stabilize specific interactions, promoting particular conformations of the receptor.[210,211] Allosteric modulators also frequently display complex pharmacology. Because allosteric modulators bind at distinct sites from orthosteric ligands, they can affect the potency of orthosteric ligands as well as their signaling efficacy. Additionally, they may display "probe dependence," where the effects of an allosteric modulator may differ depending on the specific orthosteric ligand bound to the receptor.[140] Allosteric modulators can be defined as positive (enhance orthosteric agonist binding/activity), negative (diminish it), silent (no effect on the orthosteric agonist), or biased (promote a biased response to an orthosteric agonist).[212]

At the β_2AR, allosteric modulators have been found to bind to at least three separate sites and promote distinct active or inactive conformations of the receptor, showcasing the druggability of diverse allosteric sites and the manifold modulatory effects associated with them[210,211,213] For example, the negative allosteric modulator AS408 binds between transmembrane helix 3 and 5 of the β_2AR, stabilizing key residues in an inactive conformation.[211] On the other hand, the negative allosteric modulator CMPD-15 binds to an allosteric site in the intracellular surface of the β_2AR. There, in addition to stabilizing an inactive conformation of the receptor, it also physically interferes with transducer binding, resulting in a dual mechanism of receptor inhibition.[213]

There is also one solved structure of a positive allosteric modulator bound to the β_2AR stabilizing an active conformation capable of signaling through both G-protein and β-arrestin.[210] CMPD-6 binds to the base of transmembrane helix 3 and 5 and promotes an active conformation by stabilizing key activation switches, including the formation of a helix within intracellular loop 2, and stabilizing a conserved DRY motif found in many GPCRs.[210] Interestingly, CMPD-6 also displays unique cooperativity with the arrestin biased ligand carvedilol, suggesting that analogs selectively biased towards potentiating β-arrestin signaling could be developed for this site as well.[140] Besides CMPD-6, additional allosteric modulators with biased properties have been identified for the β_2AR. These include DFPQ derivatives, isolated from high throughput screening in cells, and

CMPD-36, 37, and 42, isolated from computational screening of allosteric ligands for the β_2AR.[214,215] In general these compounds inhibit β-arrestin signaling by orthosteric ligands while permitting G-protein signaling, thus biasing receptor signaling towards G-protein. Computational docking and mutagenesis studies suggest that these compounds bind to a range of allosteric sites on the β_2AR. However, their structures have not yet been solved, so the key molecular switches they stabilize are yet to be determined (Figure 6B).

In contrast to the orthosteric site, which is well-defined with respect to its location and ligand binding, allosteric sites across the receptor surface vary greatly. Due to their diversity, allosteric modulators offer several advantages over traditional orthosteric drugs. First, allosteric sites often have the opportunity to encode greater receptor subtype specificity. Many receptors for the same agonist, e.g., adrenergic receptors, have tight evolutionary constraints on the orthosteric site, so drugs targeting that site will likely bind to multiple receptor subtypes. As a result of the reduced evolutionary constraints at allosteric sites, which do not need to maintain specificity for binding to the endogenous orthosteric ligand, allosteric modulators may be designed to yield subtype-specific drugs. Additionally, allosteric modulators can regulate the binding and signaling efficacy of orthosteric ligands in multiple ways, achieving signaling outcomes that would not be possible by targeting the orthosteric site alone. This last point is particularly important: as a consequence of binding to sites on the receptor distinct from the orthosteric site, allosteric modulators may be able to stabilize biased receptor signaling conformations that may be difficult or impossible to achieve with an orthosteric ligand.[208] For example, a β-arrestin-biased allosteric modulator for the neurotensin receptor, ML314, and its derivative, SBI-553, selectively antagonize G-protein signaling while attenuating addictive behaviors via β-arrestin-dependent signaling processes.[216] This relies on its ability to sterically interfere with $G\alpha_q$-protein coupling, but not coupling to GRK or β-arrestin.[217,218]

Signaling from Subcellular Compartments

Nanodomains—There is increasing evidence that signals generated by receptors are confined to nanometer-sized domains, referred to as nanodomains (Figure 7).[219] For example, functionally divergent pools of βARs contribute to cAMP compartmentalization to fine-tune physiological cardiac functions such as contractility. Early work in the 1980s proposed that cAMP microdomains are regulated by tethered PKA in cardiomyocytes (Figure 7C).[220] With the development of cAMP and EPAC fluorescence resonance energy transfer (FRET)-based biosensors, monitoring second messengers in subcellular locations became possible. Cardiac myocytes transfected with PKA FRET biosensors localized to the sarcoplasmic reticular and myofilament sites revealed heterogeneity in kinetics and amplitude of cAMP signaling.[221] cAMP microdomains have been shown to influence cardiac contractility and dysregulation of these domains has been implicated in heart failure (Figure 7D).[222] Recently, engineered nanoruler FRET biosensors have identified cAMP nanodomains at the GLP-1R and β_2AR, suggesting that these domains are the fundamental units of signaling in the cell.[223] These cAMP domains are dynamic entities, localized to specific subcellular regions with tight regulation of cAMP levels that are distinct from bulk cytosolic cAMP. In general, these domains are regulated by AC, phosphodiesterases (which

break down cAMP), and scaffold proteins such as A-kinase anchoring proteins (AKAP), which contribute to this localization.

Location bias—Not long after the discovery that GPCRs could signal from distinct nanodomains, it was realized that this signaling could differ between subcellular compartments, a phenomenon now commonly referred to as location bias. It is now appreciated that many receptors, such as β_1AR, β_2AR, and AT_1R, display location bias from different subcellular locations such as the nucleus, endosomes, and Golgi, which result in physiologically distinct outcomes.[224–226] In 1998, the first evidence of receptor internalization being required for MAPK activation was demonstrated at the β_2AR in the Lefkowitz Lab. In that study, it was shown that expressing β-arrestin or dynamin dominant negative mutants in HEK293 cells inhibited MAPK activation but did not limit the receptor's ability to couple to G proteins.[227] Subsequent work supported that receptor internalization did not simply terminate signaling, but promoted signaling by G-proteins from endosomes.[228] In 2009, three independent groups showed that thyroid stimulating hormone, parathyroid hormone, and the sphingosine-1 phosphate receptors continue to signal after their internalization.[229–231] Inhibition of internalization resulted in the ablation of cAMP signaling, demonstrating that G protein signaling can occur from endosomes. Subsequent work showed that other $G\alpha_s$-coupled receptors, such as internalized β_2ARs, increase intracellular cAMP levels, which was subsequently shown to promote CREB-dependent transcription.[224,232]

A few studies have probed the consequences of location-biased signaling in physiologically relevant cell types, such as cardiomyocytes, in health and disease.[222] β_1ARs are found throughout the cardiomyocyte, while the β_2AR and β_3AR subtypes are solely expressed in the T-tubules,[233] and cAMP generation from receptors at T-tubules activates a subset of PKA anchored in their vicinity[234] (Figure 7A). Receptors are localized to other subcellular compartments such as the nuclear membrane, where α_1ARs,[235,236] AT_1Rs,[237] ETARs,[238] and βARs[129] are present in cardiomyocytes. Nuclear α_1AR translocates from the nucleus to the caveolae to induce ERK signaling [129,235] and AT_1Rs in the nucleus has been observed to regulate N-κB transcription.[237] β_1ARs and β_2ARs are also found on nuclear membranes of cardiomyocytes where they activate $G\alpha_s$ and other signaling partners, which alter transcriptional networks upon receptor activation.[129] Another important subcellular compartment is the Golgi/sarcoplasmic reticulum, where a pool of β_1ARs resides.[225] The accessibility of different ligands to this pool of receptors depends on their membrane permeability, which can result in distinct physiological responses. The organic cation transporter subtype 3 (OCT3), has been shown to facilitate the uptake of membrane-impermeable catecholamines across the plasma membrane to the nucleus and Golgi.[235] For example, OCT3-mediated transported ligands can regulate the sarcoplasmic reticulum localized β_1AR and regulate contractility through local PKA-mediated phosphorylation of phospholamban (Figure 7E). The function of β_1AR localization has been further elucidated in intact zebrafish hearts, where Golgi-localized β_1AR cAMP production promotes lusitropy (through PLB/SERCA), while plasma membrane-localized β_1AR mediates PKA phosphorylation of RyR2 and troponin I to promote inotropy.[239] Further work with drugs

with different membrane permeability may aid in uncovering the physiological significance of subcellular signaling in cardiomyocytes.[240]

Conclusions

Our understanding of cardiovascular GPCRs has had a profound impact on the development of modern-day cardiovascular therapies. Over the past century, researchers have made many key discoveries on the nature of GPCR signaling, elucidating multiple mechanisms of drug action. With the substantial growth and excitement around cardiovascular GPCRs, these receptors are still some of the top targets in drug discovery. Additional understanding of receptor structures and receptor dynamics, will improve screening approaches, including those that target new allosteric sites on receptors and incorporate novel computational and artificial intelligence approaches. These could also be used to guide the development of drugs that have bias between heterotrimeric G-proteins and β-arrestins as well as having specific patterns of location bias. With new data sets, such as those from single-cell sequencing and other approaches, it should also be possible to identify novel therapeutic targets for cardiovascular disease. Combining these approaches, across pharmacology, physiology, structure, and computational biology, should lead to an exciting century-to-come of new GPCR-targeting therapies in cardiovascular disease.

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Non-standard Abbreviations and Acronyms

GPCR	G protein-coupled receptor
cAMP	cyclic adenosine monophosphate
AC	adenylyl cyclase
GTP	guanosine triphosphate
GDP	guanosine diphosphate
βAR	β-adrenergic receptor
GRK	GPCR kinases
AT1R	angiotensin II type 1 receptor
HF	heart failure
ATP	adenosine triphosphate

PKA	protein kinase A
PIP_2	phosphatidylinositol 4,5-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
DAG	diacylglycerol
SR	sarcoplasmic reticulum
PKC	protein kinase C
RGS	regulators of G protein signaling
GAPs	GTPase activating proteins
GLP-1	Glucagon-like peptide-1
GLP-1R	The GLP-1 receptor
GIP	glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
PDB	protein data bank
Fab	fragment antigen-binding
NMR	nuclear magnetic resonance
EPR	electron paramagnetic resonance
DEER	double electron–electron resonance
DRY	aspartic acid-arginine-tyrosine
FRET	fluorescence resonance energy transfer
OCT3	organic cation transporter subtype 3
PLC-β	phospholipase C-β
ERK	extracellular signaling-related kinases
AKAP	A-kinase anchoring protein
PDE	phosphodiesterase
PLB	hospholamban
SERCA	Sarcoendoplasmic Reticulum Calcium ATPase

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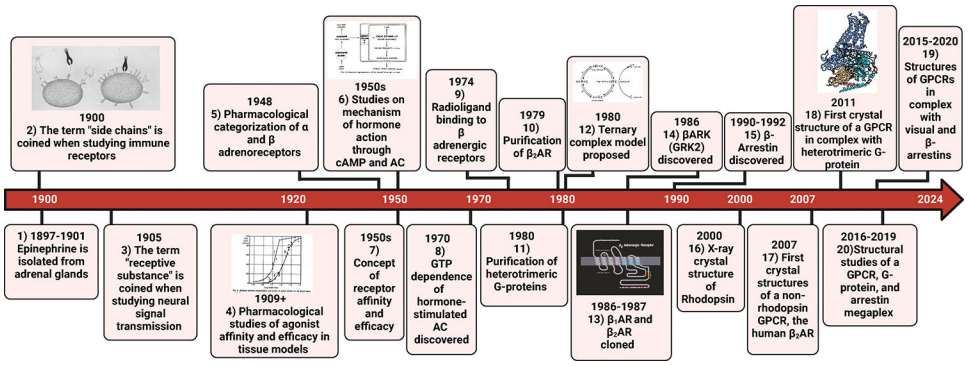

Figure 1: Notable Ligand, Receptor, and Transducer Discoveries.

Over the past century, there have been many discoveries from ligands to transducers. Here we have highlighted key studies that have contributed to our knowledge of GPCRs. Below are the corresponding publications for each discovery. Images were reproduced with permission from reference #'s

1. 1897–1901: Epinephrine is characterized and isolated from adrenal glands.[4,241] **2.** 1900: The term "side chains" is coined when studying immune receptors.[7] **3.** 1905: The term "receptive substance" is coined when studying neural signal transmission.[9] **4.** 1909+: Pharmacological studies of agonist affinity and efficacy in tissue models.[15–18] **5.** 1948: Pharmacological categorization of α and β adrenoreceptors.[242] **6.** 1950s: Studies on the mechanism of hormone action through cAMP and Adenylyl Cyclase (AC). [21,23,243] **7.** 1950s: Concept of receptor affinity and efficacy.[19,20] **8.** 1971: GTP dependence of hormone-stimulated AC discovered.[24–26] **9.** 1974: Radioligand binding of β adrenergic receptors.[31–33] **10.** 1979: Purification of the β_2AR.[36] **11.** 1980: Purification of heterotrimeric G-proteins.[27,28] **12.**1980: Ternary complex proposed.[35] **13.** 1986–8: β_1AR and β_2AR cloned.[37,244,245] **14.** 1986: βARK (GRK2) discovered.[38] **15.** 1990–1992: β-arrestins discovered.[39,40] **16.** 2000: X-ray crystal structure of Rhodopsin.[246] **17.** 2007: First crystal structures of a non-rhodopsin GPCR, the human β_2AR.[191,192] **18.** 2011: First crystal structure of a GPCR in complex with heterotrimeric G-protein.[193] **19.** 2015–2020: Structures of GPCRs in complex with visual and β-arrestins.[247–249] **20.** 2016–2019: Structural studies of a GPCR, G-protein, and arrestin megaplex. [250,251]

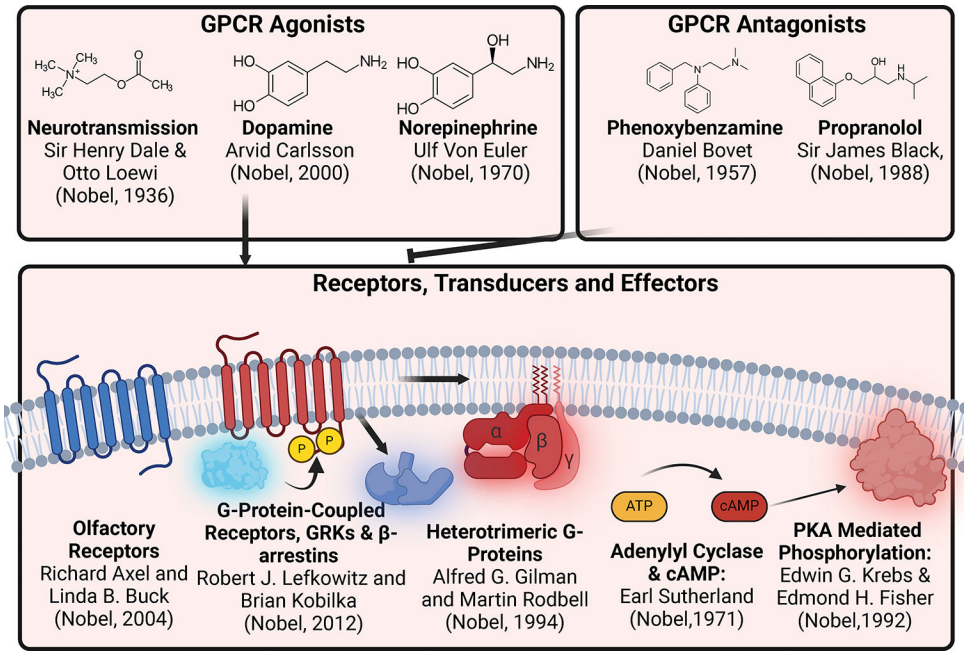

Figure 2: History of Nobel Prize winning GPCR Discoveries

The discoveries of components of the GPCR signaling system, ranging from ligands, receptors, and transducers have resulted in numerous Nobel Prizes being awarded. These include the development of clinically important agonists and antagonists, and basic discoveries related to the signaling mechanisms of GPCRs and their transducers. Names and Nobel Prizes were collected from the Nobel Foundation website (https://www.nobelprize.org/prizes/lists/all-nobel-prizes/)

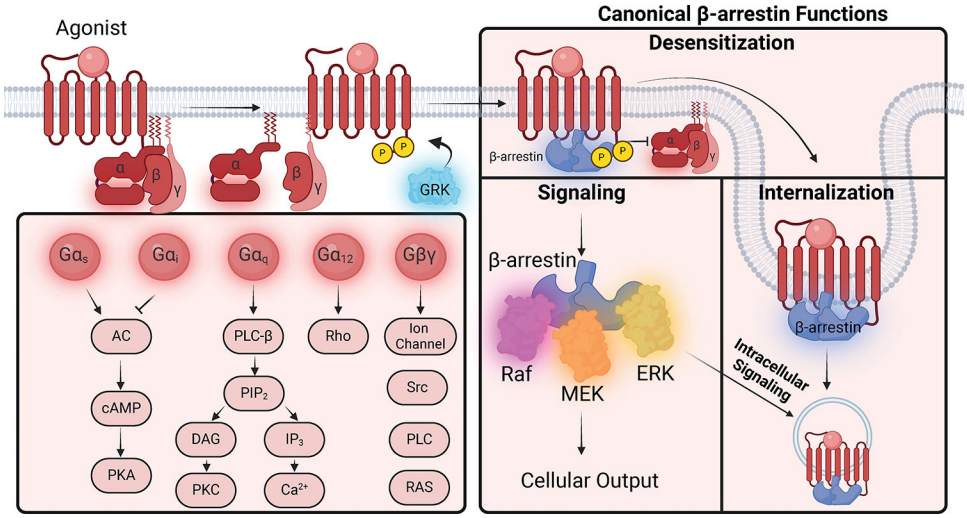

Figure 3: GPCR signaling via G proteins, GRKs and β-arrestins.

Upon agonist stimulation, heterotrimeric G-proteins (Gα, Gβ, and Gγ) are recruited to the receptor and there is guanine nucleotide exchange of GTP for GDP. The Gα-GTP subunit dissociates from the G$\beta\gamma$ transducer, and both signal to diverse downstream effectors. Gα has four distinctive families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12/13}$). GRKs phosphorylate the intracellular domains of the GPCR, which promote tight binding of β-arrestins. β-arrestins have three canonical functions: receptor desensitization, internalization, and signaling. In addition, there is intracellular signaling of β-arrestins from endosomes. GRK, G-protein receptor kinase; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; AC, adenyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA; Protein kinase A; PLC- β, phospholipase C- β; IP_3, inositol trisphosphate; PIP_2 phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PKC, Protein kinase C; ERK, extracellular signaling-related kinases

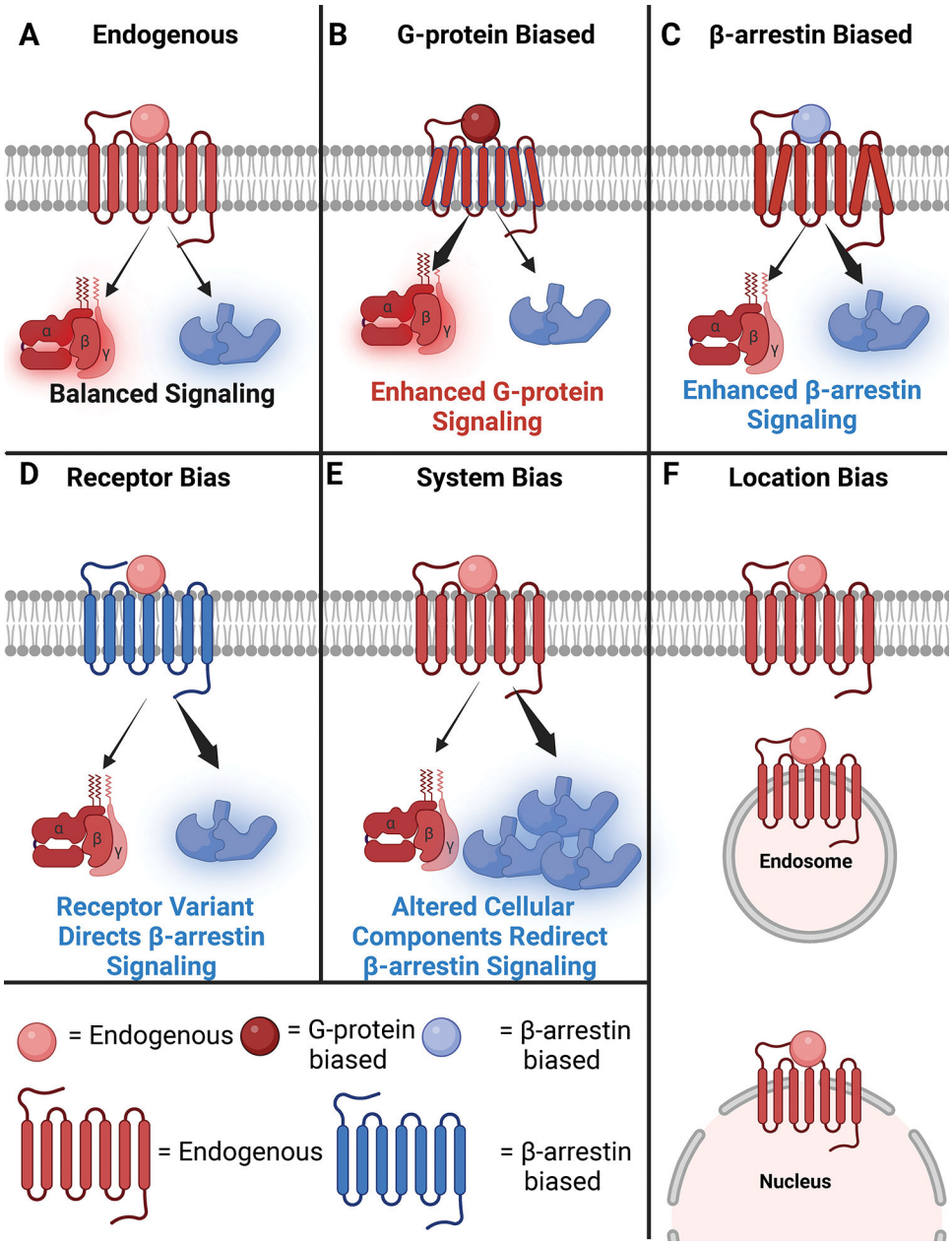

Figure 4: Biased Signaling of GPCRs.

(**A**) Reference/endogenous agonists binding to receptors can signal through two different pathways, G-proteins and β-arrestin. (**B**) Ligand bias promotes the receptor:transducer complex to adopt certain conformations that bias the signaling through (**B**) G-proteins or (**C**) β-arrestin. (**D**) Biased receptors may have altered phosphorylation sites on their C-tail or other mutations that may bias signaling toward β-arrestin instead of G-proteins despite being stimulated with an endogenous agonist. (**E**) System bias occurs when there is a differential expression of signaling components. For example, some cells express different isoforms of GRK and β-arrestin and may bias signaling through β-arrestin. (**F**) Location bias refers to receptors promoting signaling from distinct intracellular locations, such as from endosomes, the nucleus, or the plasma membrane.

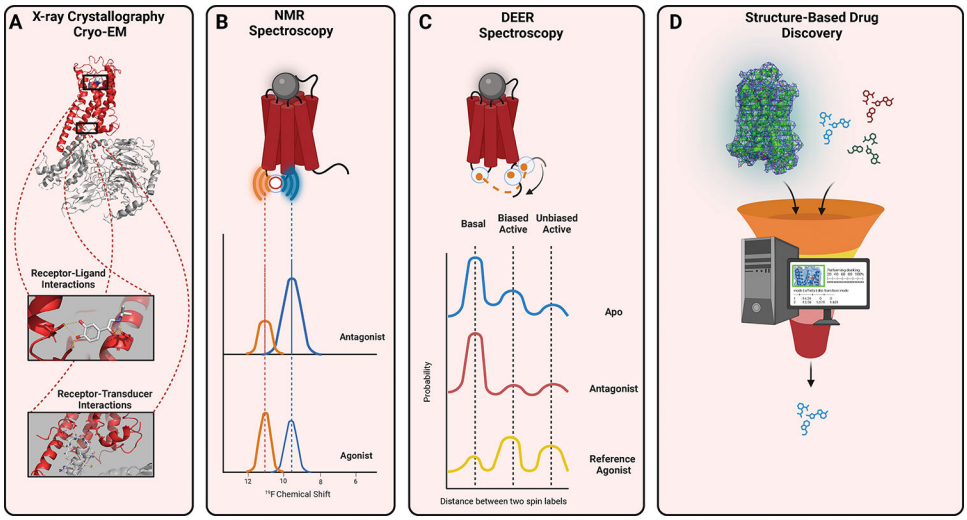

Figure 5: Structural studies of GPCRs.

(**A**) X-ray crystallography and CryoEM provide static structures of GPCRs. With improvements in cryoEM technology and workflow, cryoEM can now provide high-resolution data on both receptor-ligand and receptor-transducer interactions, making it particularly well-suited to the study of GPCRs. (**B**) NMR Spectroscopy with probes such as ^{19}F can provide information on dynamics and conformational ensembles by measuring changes in the local environment of individually labeled probes. (**C**) EPR spectroscopy techniques such as DEER spectroscopy with two spin-label probes can provide information on distances, conformations, and their relative populations. (**D**) Structure-Based Drug Discovery techniques use receptor structures to allow molecular docking and other approaches to screen compounds that bind to a desired receptor conformation.

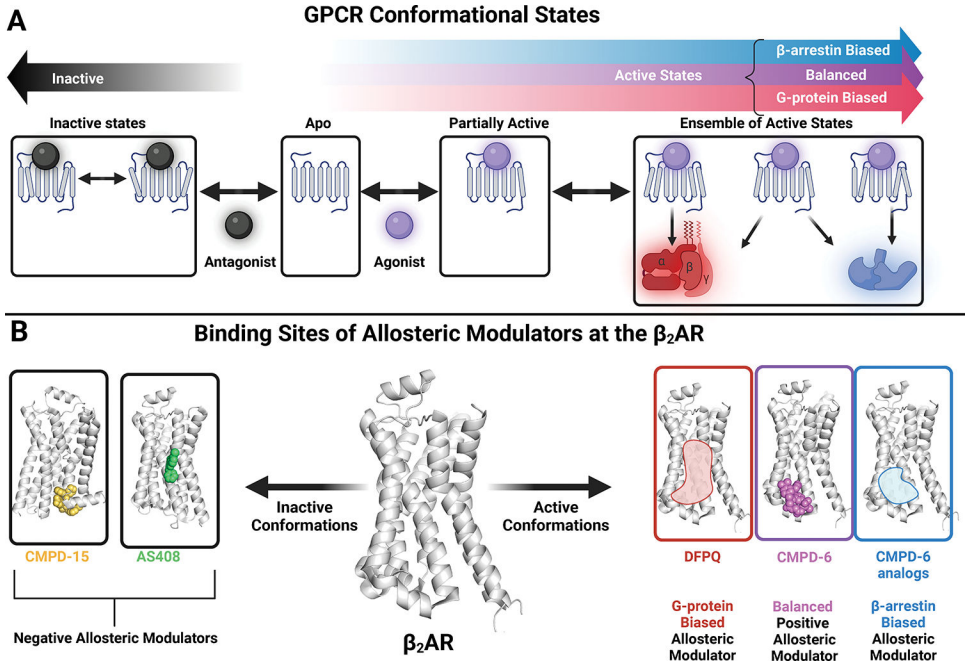

Figure 6: Allosteric Modulation of GPCRs.

(**A**) GPCR Conformational States. Receptors in the Apo (no ligands bound) state are largely in inactive conformations but can be stabilized by orthosteric agonists and antagonists in a variety of different active or inactive states. Active states are characterized by an opening of the transducer binding pocket. This pocket is closed to different degrees in inactive states. Orthosteric agonists can activate both G-protein and β-arrestin pathways or be biased towards one pathway. (**B**) Allosteric modulators bind to topographically distinct sites from the orthosteric site that can modulate receptor conformation. Allosteric modulators can either enhance agonist affinity and/or efficacy (positive allosteric modulators) or diminish the agonist affinity and/or efficacy (negative allosteric modulators). In addition, positive allosteric modulators can stabilize specific receptor conformations that promote bias towards G-protein or β-arrestin. Illustrated are the binding sites of allosteric modulators at the β_2AR and the conformations they promote. The negative allosteric modulators of the β_2AR, CMPD-15, and AS408, bind to distinct sites on the β_2AR. CMPD-6, a balanced positive allosteric modulator, binds to another distinct site. Allosteric modulators can also display biased properties such as difluorophenyl quinazoline derivatives (DFPQ) which biases the β_2AR towards G-protein signaling. The binding pose of DFPQ compounds has not been experimentally solved, but they are believed to bind close to the CMPD-6 binding site. β-arrestin biased analogs of CMPD-6 have also been proposed due to CMPD-6's interactions with the β-arrestin biased orthosteric ligand carvedilol. Compound structures obtained from the Protein Data Bank (PDB) : CMPD-15: 5X7D, AS408: 60BA, CMPD-6: 6N48.

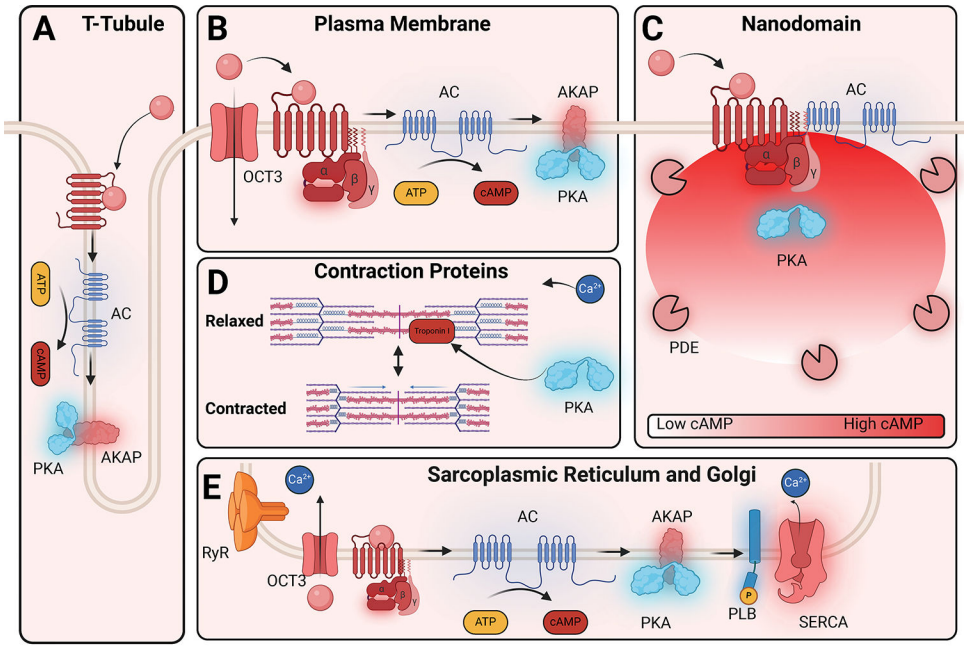

Figure 7: Location Bias in Cardiac Myocytes.

(**A**) In healthy cardiac tissue, βARs are found in T-tubules, which when stimulated with epinephrine/norepinephrine activate βAR-AC-PKA. PKA is tethered to intracellular compartments by AKAPs. PKA phosphorylates the RyR, inducing calcium release and resulting in increased cardiac contractility. (**B**) βARs at the plasma membrane enhance contractility through βAR-AC-PKA activation. (**C**) Recent work has evaluated cAMP nanodomains with different $G\alpha_s$-mediated receptors. Nanodomains are membraneless compartments that enhance or sequester signaling molecules. For example, PDE is involved in regulating the size and shape of cAMP nanodomains, whereas AKAP (AKAP not pictured) serves as a PKA scaffolding protein to sequester cAMP signaling. (**D**) Contractility is promoted by Ca^{2+} binding to troponin C in addition to PKA mediated-phosphorylation of contractile proteins such as troponin I (**E**) OCT3 is found on the plasma membrane and the Golgi and facilitates the transportation of norepinephrine/epinephrine into the cell to promote Golgi-βARs-AC-PKA signaling. Unlike the pool of PKA found at the plasma membrane and T-tubules, Golgi PKA activates phospholamban and forms an inhibitory complex with SERCA. This reduces available Ca^{2+} and increases the rate of relaxation. βARs, β-adrenergic receptor; AC, adenyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA; Protein kinase A; AKAP; A-kinase anchoring protein; PDE, phosphodiesterase; PLB, phospholamban; SERCA, Sarcoendoplasmic Reticulum Calcium ATPase; OCT3, monoamine transporter; RyR, Ryanodine Receptor

Table 1.

Notable FDA-approved drugs, physiological effects, and cardiovascular clinical trials of drugs targeting the β-adrenergic, angiotensin II type 1, and incretin receptors.

Receptor Activity	Proximal Effectors	FDA-approved Agents	Cardiovascular Effects	Notable Clinical Trials
β-adrenergic receptors (β_1AR and β_2AR) antagonists	$G\alpha_s$, $G\alpha_i$, β -arrestin 1/2	**Beta blockers:** Acebutolol, Atenolol, Betaxolol, Bisoprolol, Carvedilol, Labetalol, Metoprolol, Nadolol, Nebivolol, Pindolol, Propranolol, Timolol	- Heart rate control - Negative inotropy - Reduction in myocardial oxygen demand - Reduces BP - Treatment of heart failure	**Heart Failure with Reduced Ejection Fraction:** 1993: Metoprolol in Dilated Cardiomyopathy (*Metoprolol*)[53] 1996: US Carvedilol HF Study (Carvedilol)[54] 1999: MERIT-HF (Metoprolol)[56] 1999: CIBIS-II (Bisoprolol)[55] 2001: COPERNICUS (Carvedilol)[57]
Angiotensin II Type 1 Receptor (AT_1R) antagonists	$G\alpha_q$, $G\alpha_i$, β -arrestin 1/2	**ACE-I:** Benazepril, Captopril, Enalapril, Fosinopril, Lisinopril, Moexipril, Perindopril, Quinapril, Ramipril, Transolapril **ARB:** Candesartan, Eprosartan, Irbesartan, Losartan, Olmesartan, Telmisartan, Valsartan	- Reduces BP - Treatment of heart failure - Renoprotection by decreasing glomerular filtration	**Heart Failure with Reduced Ejection Fraction:** 1987: CONSENSUS (Enalapril)[157] 1991: V-HeFT II (Enalapril)[252] 1991: SOLVD-T (Enalapril)[253] 1992: SOLVD-P (Enalapril)[254] 1997: ELITE (Losartan / Captopril)[158] 2001: Val-HeFT (Valsartan)[255] 2003: CHARM-Alt (Candesartan)[256] 2014: PARADIGM-HF (Sacubitril-Valsartan / Enalapril)[159]
Glucagon-like peptide 1 receptor (GLP-1R) agonists	$G\alpha_s$, $G\alpha_q$, $G\alpha_i$, β -arrestin 1	Dulaglutide, Exenatide, Liraglutide, Lixisenatide, Tirzepatide (dual GLP-1R and GIPR agonist)	- Weight loss - Glycemic control - Reduction in major cardiovascular events - Improvement in heart failure	**Major adverse cardiovascular events (Nonfatal myocardial infarction, nonfatal stroke, or cardiovascular death):** 2021 Meta-analysis of eight clinical trials in diabetes (Lixisenatide, Liraglutide, Semaglutide, Exenatide, Albiglutide, Dulaglutide, Efpeglenatide)[172] 2023 SELECT – Obesity and CAD without diabetes (Semaglutide)[173] **Heart Failure with Preserved Ejection Fraction:** 2023 STEP-HFpEF – Obesity without diabetes (Semaglutide)[174]