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Technology Review

Emerging Concepts of Guanine Nucleotide-Binding Protein-Coupled Receptor (GPCR) Function and Implications for High Throughput Screening

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Abstract: Guanine nucleotide binding protein (G protein) coupled receptors (GPCRs) comprise one of the largest families of proteins in the human genome and are a target for 40% of all approved drugs. GPCRs have unique structural motifs that allow them to interact with a wide and diverse series of extracellular ligands, as well as intracellular proteins, G proteins, receptor activity-modifying proteins, arrestins, and indeed other receptors. This distinctive structure has led to numerous efforts to discover drugs against GPCRs with targeted therapeutic uses. Such "designer" drugs currently include allosteric regulators, inverse agonists, and drugs targeting hetero-oligomeric complexes. Moreover, the large family of orphan GPCRs provides a rich and novel field of targets to discover drugs with unique therapeutic properties. The numerous technologies to discover GPCR drugs have also greatly advanced over the years, facilitating compound screening against known and orphan GPCRs, as well as in the identification of unique designer GPCR drugs. Indeed, high throughput screening (HTS) technologies employing functional cell-based approaches are now widely used. These include measurement of second messenger accumulation such as cyclic AMP, calcium ions, and inositol phosphates, as well as mitogen-activated protein kinase activation, protein–protein interactions, and GPCR oligomerization. This review focuses on how the improved understanding of the molecular pharmacology of GPCRs, coupled with a plethora of novel HTS technologies, is leading to the discovery and development of an entirely new generation of GPCR-based therapeutics.

Introduction

GUANINE NUCLEOTIDE BINDING protein (G protein) coupled receptors (GPCRs) are critical proteins that provide much of the diversity in cell-to-cell communication. These heptahelical proteins are expressed on the cell surface and bind almost all of the known neurotransmitters and hormones released synaptically or that are secreted into the circulatory system controlling organ function.[1] The ligand–GPCR binding reaction initiates a cascade of intracellular events, including changes in levels of second messengers, ionic conductance, and a host

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; cAMP, cyclic AMP; CFTR, cystic fibrosis chloride channel; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; ERK, extracellular signal-regulated kinase; FRET, fluorescence resonance energy transfer; G protein, guanine nucleotide binding protein; GABA, γ-aminobutyric acid; GPCR, guanine nucleotide binding protein coupled receptor; GRK, guanine nucleotide binding protein receptor kinase; GTPγS, guanosine 5′-(γ-thio)triphosphate; IP_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; RAMP, receptor activity-modifying protein; VPAC, vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide.

of other molecular events that alter cellular activity. Since GPCRs couple neurotransmitters and hormones with their target cells, they are thus essential for normal physiological function. Consequently, dysfunctional GPCR activity is involved in the etiology of several diseases and disorders.

Because of their central role in cellular communication, GPCRs have historically served a fundamental function in modern pharmacological research. They have also been targets for the discovery of many drugs developed by the pharmaceutical industry. By one estimate,[2] over 40% of marketed drugs target GPCRs.[3] When one considers that the human genome expresses genes for between 800 and 1,000 different GPCRs[4] and marketed drugs target less than 50 GPCRs, it is evident that the field of GPCR drug discovery and development will grow in the years ahead.

The primary approach used to discover GPCR drugs today involves the use of automated HTS assays principally since approaches employing crystallography and rational medicinal chemistry—the basis of much of the drug design and discovery against soluble enzymes—cannot be easily employed to discover drugs against GPCRs. Generally, GPCRs resist purification in native, functional conformations, and purification is a prerequisite for crystallographic analysis. Indeed, with the exception of rhodopsin, the crystal structures of no other GPCR has been reported to date.

Over the last decade, HTS technologies to discover GPCR drugs have greatly expanded such that they can be readily adapted to automated fluid dispensing systems and microtiter plate detectors. They are also more sophisticated in terms of measuring the response of the GPCR in a cellular context. Indeed, cell-based assays have become necessary as numerous GPCRs possess no identified endogenous ligand and are thus "orphan" in nature. Orphan GPCRs could provide a large area of expansion of GPCR drug discovery, yet require novel technologies for implementation in drug discovery. It is thus not feasible to identify compounds against orphan GPCRs in the same fashion as has historically been done for the known liganded GPCRs. Furthermore, the possibility of developing novel drugs targeting GPCRs, such as inverse agonists, allosteric modulators, and drugs addressing GPCR heterodimers, all require novel HTS technologies.

This review attempts to focus on those new technologies, many of which are allowing for the development of an entirely new generation of GPCR-based therapeutics.

GPCR Structure and Function

The GPCR protein family possesses unique structural motifs rendering them unique among protein superfamilies.[5] This distinctive motif is used to assign genes and their predicted gene products as putative members of the GPCR superfamily. GPCRs are single-chain polypeptides consisting of approximately 300 amino acids, with alternating regions of hydrophilicity and hydrophobicity. Being integral cell membrane proteins, the hydrophobic regions define the transmembrane regions of the receptors. The amino- and carboxyl-termini and the intervening extra- and intracellular loops are generally more hydrophilic. The pattern of alternating hydrophilicity and hydrophobicity thus defines the archetypal seven-transmembrane structure of GPCRs. The amino-terminus is orientated to face the extracellular milieu, while the carboxyl-tail faces the cell cytoplasm.

The hydrophobic, transmembrane regions provide the structural scaffold to confine the receptors in their functional conformations. The hydrophilic regions including the extracellular and intracellular loops and the carboxyl-terminus provide contact regions for ligand binding and G protein coupling, respectively. Rather than being static, GPCRs undergo subtle conformational changes in these transmembrane regions when ligands bind to the extracellular domains, opening up intracellular domains to contact G proteins and thus initiate signal transduction.

Ligand Binding Domains

GPCRs are classified on the nature of the ligands with which they interact. Class 1 includes rhodopsin-like receptors (for which the β-adrenergic receptor is prototypic). Ligands that activate these receptors include biogenic amines, chemokines, prostanoids, and neuropeptides. Class 2 includes the secretin-like receptors and are activated by ligands such as secretin, parathyroid hormone, glucagon, calcitonin gene-related peptide (CGRP), adrenomedullin, and calcitonin. Class 3 includes metabotropic glutamate receptor-like and calcium sensing receptors.[6]

Much of what is known about the nature of ligand binding to GPCRs derives from seminal studies conducted on the β-adrenergic receptor, a receptor that binds small molecule ligands in a unique hydrophobic extracellular pocket.[7–9] As the endogenous ligands (in the case of the β-adrenergic receptor, the endogenous ligands are epinephrine and norepinephrine) are small, they insert into these binding pockets and interact with sites deep within the core of the structure to induce activation. GPCRs recognizing larger endogenous ligands, such as peptides, bind their ligands differently.[10] Here, peptides interact with recognition sites in the extracellular loops of receptors as well as with residues in the hydrophobic pocket.[11–13] To take an example, the endogenous ligand of the κ-opioid receptor is dynorphin A, which is 18

amino acids in length.[14] This peptide binds to charged residues in the second extracellular loop in a region essential for the specificity of dynorphin A to bind to this receptor.[15–17] This was shown in mutagenesis studies exchanging the second extracellular loop of the κ receptor with the μ-opioid receptor,[15] by nuclear magnetic resonance analysis of the second extracellular loop,[16] and also by modifying the carboxyl-terminal amino acids of dynorphin A to identify the address signal of the peptide for the receptor.[17] Similarly, the 14- and 28-amino acid forms of somatostatin bind to extracellular loops of the somatostatin receptor subtypes I and II to produce biological responses.[12] Targeting of recognition sites to GPCR extracellular loops is critical for creating high affinity of GPCRs for peptides that are too large to intrude entirely into hydrophobic pockets that bind small molecules. Clearly, ligands binding to extracellular loops are expected to induce different conformational changes in the receptor upon binding than small molecules that interact with residues deep within the receptor core.

Interestingly, the multiplicity of recognition sites in peptide receptors may reveal the diversity of ligand interaction with the same GPCR in order to elicit different functional effects.[10] For example, the μ-opiate receptor is a target for the biological actions of the endogenous peptides enkephalin and β-endorphin.[14] β-Endorphin is a large agonist, being 31 amino acids in length. The μ-opiate receptor also binds small molecules, such as morphine, that also stimulate the receptor and that act as potent analgesics. Although morphine and endorphin are both agonists and produce similar cellular responses via activation of μ receptors, they produce differing responses *in vivo*. Thus, morphine exhibits highly addictive properties, whereas neither enkephalin nor β-endorphin causes dependence when administered centrally. Such functional differences may be due to morphine and endorphin inducing different conformational changes in the receptor to active distinct downstream signaling events. This is further indicated by studies showing that morphine produces little desensitization or internalization of μ-opiate receptors[18] and is weak in inducing translocation of the regulatory protein, β-arrestin, to the receptor.[19] This is in contrast to other μ agonists, which suggests that morphine binds to the receptor differently than other agonists.

Different conformations induced by ligands are not restricted to peptide receptors as suggested by recent studies on the β-adrenergic receptor.[20] In these studies, a fluorescent tag was inserted into a cysteine residue at position 265 in the receptor at the cytoplasmic termination point of transmembrane 6, which is in a region near the G protein coupling domain of this receptor. Quenchers were inserted into other amino acids in the receptor. Using fluorescence resonance energy transfer (FRET), it was thus possible to directly measure changes in interaction of cysteine-265 with different residues in the receptor as a reflection of conformational change in the receptor in response to agonist binding. It was found that upon agonist binding to the receptor, the region of the receptor encompassing cysteine-265 rotated towards regions in transmembrane 5, suggesting a reorientation towards a more hydrophobic region of the receptor and its environment. Importantly, it was found that the conformational changes that were induced by a series of agonists were proportional to their efficacy in activating adenylyl cyclase.

Of interest are more recent findings[21] by the same group in which fluorescent tags were inserted into cysteine-265 and the C-terminal end of the β-adrenergic receptor, a region not only involved in G protein coupling but association with other allosteric regulators, such as the arrestins and protein kinases such as extracellular signal-regulated kinases (ERKs), as described more extensively below. In these studies, it was found that when the receptor is quiescent, the C-terminal tail maintains an extended unstructured conformation. When agonists bind to the receptor a FRET response was detected consistent with the C-terminal tail reorientating to a position closer to transmembrane 6, in effect bending back onto the core of the receptor. Agonists that induce an association of the receptor with ERKs produce a different fluorescent response than those that do not affect receptor–ERK interactions. These results, in the context of those of the previous studies[20] examining interactions of regions of transmembrane 5 and transmembrane 6, suggest that a myriad of conformational perturbations occur when agonists bind to GPCRs and that the type of three-dimensional change induced by an agonist could reflect subtle functional differences that one ligand may induce versus another.

The differential ability of ligands targeting the same receptor to induce distinct biological effects opens up new avenues for the discovery of novel drugs with optimal therapeutic actions. As described below, ligands including partial agonists, inverse agonists, and allosteric regulators are currently important novel classes of drugs, capable of inducing desired pharmacological effects distinct from classical agonists. These ligands may induce different conformational changes on GPCRs than classical full agonists and, as a consequence, produce different pharmacological actions. By implication, screening technologies allowing one to monitor these diverse activation processes could be useful for discovery of these novel types of GPCR drugs.

GPCRs and G Proteins

The intracellular domains of GPCRs contain several contact regions responsible for receptor coupling to sig-

nal transduction systems. The regions most prominent in GPCRs for coupling are the second and third intracellular loops as well as the C-terminal tail. Predominant among the intracellular effector molecules contacted by these regions are the G proteins, which link GPCRs to second messenger systems, such as adenylyl cyclase, phospholipases, and ionic conductance channels.[22]

G proteins interact with cell surface receptors and exhibit inherent GTPase catalytic activity.[23] The G protein superfamily consists of heterotrimeric complexes of distinct α, β, and γ subunits. There are thought to be 18 Gα, five Gβ, and 11 Gγ subunits, collectively creating a very large number of distinct heterotrimeric complexes.[24] These different heterotrimers have significant specificity with regards to both the GPCRs with which they interact and the cellular effector systems that they regulate.[23,25,26] The α subunit of G proteins contains the GTPase catalytic activity as well as many of the receptor contact sites.[23] Agonist binding to the GPCR promotes a conformational change to induce coupling with the G protein. The GPCR/G protein interaction accelerates catalysis of GTP to GDP, providing energy needed for dissociation of the α from the $\beta\gamma$ subunits (in general, $\beta\gamma$ subunits are tightly associated and do not easily dissociate). The free α and $\beta\gamma$ subunits then interact with second messenger systems and ionic conductance channels as well as other cellular effectors.

The G proteins create the diversity of functions of GPCRs.[26,27] Thus, $G\alpha_s$ is known to couple GPCRs to adenylyl cyclase to stimulate formation of the second messenger cyclic AMP (cAMP). Once formed, cAMP in turn stimulates a family of cAMP-dependent protein kinases that modulate short-term responses such as changes in ionic conductance as well as long-term changes in cell activity via alterations in gene transcription and cell metabolism. Because nature frequently creates homeostatic systems, there also exist GPCRs that mediate the inhibition of adenylyl cyclase. These receptors, such as the α2-adrenergic, opiate, and somatostatin receptors, couple to adenylyl cyclase via $G\alpha_i$.[12,13,23,28,29] This diversity of GPCR/G protein interaction suggests that structural elements in the intracellular domains of the GPCR direct the receptors to different G proteins and thereby create the functional diversity of the receptors. Those structural elements can simply be due to unique amino acid sequences in the intracellular domains that serve as recognition sites of the receptor for the G proteins or the development of unique three-dimensional structures due to the conformational changes in the receptor following agonist binding that selectively bind the G proteins.[30–32]

While $G\alpha_i$ is critical for coupling different families of GPCRs to inhibition of adenylyl cyclase, it is also responsible for other functions of GPCRs. Therefore, subtypes of $G\alpha_i$ link GPCRs to inward rectifying K^+ channels, and stimulation of GPCR/$G\alpha_i$/K^+ channels results in hyperpolarization of cell membranes.[33–35] The modulation of the K^+ current appears to primarily involve the $\beta\gamma$ subunits linked to $G\alpha_i$.[26,27] Therefore stimulation of the same receptor/$G\alpha_i$ complex can lead to turning off the cAMP pathway and simultaneously inhibit cell firing. G proteins also couple the receptors to other cellular effector systems. Thus, $G\alpha_o$ has been shown to link GPCRs to Ca^{2+} conductance channels to regulate the influx of Ca^{2+} to cells.[36–38] Here, it is believed that dissociation of $G\alpha_o$ from $\beta\gamma$ subunits is important since $\beta\gamma$ subunits can act upon the Ca^{2+} channels to regulate conductance.[26,27] $G\alpha_o$ provides further diversity in function because it can also link GPCRs to phosphoinositol phospholipase Cβ (probably also via $\beta\gamma$ subunits), which hydrolyzes phosphatidylinositol 4,5-bisphosphate, forming *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds and opens endoplasmic IP_3-gated calcium channels, causing release of bound calcium into the cytosol.[39] Metabolic products of IP_3 also modulate cellular function including inositol 1,3,4,5-tetrakisphosphate, which acts to facilitate IP_3-mediated calcium release synergistically. Fundamentally, therefore, $G\alpha_o$ provides a critical means for GPCR regulation of cellular Ca^{2+} homeostasis. Importantly, some ligands can stimulate inhibitory GPCR (for example, opiate and somatostatin receptors)/$G\alpha_o$ complexes to inhibit Ca^{2+} conductance and influx while at the same time increasing release of intracellular Ca^{2+}. This acts as a subtle regulatory mechanism for cellular Ca^{2+} signaling, switching it from one dependent on extracellular Ca^{2+} to one dependent only on intracellular Ca^{2+}.

In addition to $G\alpha_o$, GPCRs also couple to another subfamily of G protein subunits involving $G\alpha_q$. This Gα subunit, like $G\alpha_o$, is able to link GPCRs to activation of phospholipase C to increase intracellular Ca^{2+} release to active downstream regulators such as protein kinase C. Protein kinase C activation can then lead to modulation of the mitogen-activated protein kinase (MAPK) pathway.[40] This involves stimulation of Raf, Mek, and the MAPK ERK. ERK can then phosphorylate and activate transcription factors to change gene expression and produce long-term alterations in cell activity. In fact, ERKs may be a convergent target for activation of most GPCRs since cAMP-dependent protein kinase is also involved in regulating ERKs, and therefore those GPCRs that regulate the cAMP signal transduction pathway also modulate ERK activity. Furthermore, as described below, any GPCR that is regulated by β-arrestin will also affect ERK activity.

GPCRs acting via $G\alpha_q$ can also modulate activity of growth factor receptor pathways in cells. Specifically, by mobilizing intracellular Ca^{2+}, GPCR/$G\alpha_q$ activation can lead to stimulation of the tyrosine kinase Pyk2, which can activate Src, which can cause phosphorylation and activation of the epidermal growth factor receptor.[41] This

intracellular pathway provides a mechanism for cross-talk and cross-regulation of GPCRs and growth factor receptors.

Taken together, studies over the last 20 years have revealed the G proteins can couple GPCRs to multiple cellular signaling pathways and provide diversity in the functioning of these cell surface receptors. Indeed, a single GPCR may be capable of coupling to more than one G protein. This has been shown to be the case for some somatostatin receptors as well as the δ-opioid receptors, which are able to associate with several subtypes of $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$.[11,12,42–45] This may explain how agonists at these receptors can inhibit adenylyl cyclase, as well as Ca^{2+} conductance while stimulating K^+ conductance in the same cell.

Interestingly, β_2-adrenergic receptors have an unusual duality in their coupling to G proteins.[46] While most β-adrenergic receptors in heart are of the β_1 subtype, which couples exclusively to $G\alpha_s$ to mediate stimulation of cAMP formation and cardiac inotropic effects, more recent studies have identified β_2-adrenergic receptors in heart that couple to both $G\alpha_i$ and $G\alpha_s$. $G\alpha_i$ mediates cardioprotective effects of β_2-adrenergic receptor stimulation against apoptosis in myocytes and also attenuates the inotropic effects of β-adrenergic agonists. In effect, this dual G protein coupling to the same β_2-adrenergic receptor acts as a sort of safety value, blunting excessive cardiac stimulation to protect heart tissue from damage. Such a safety mechanism may be important in the normal functioning heart but may exacerbate problems in the failing heart, as studies by He *et al*.[47] have shown that in models of heart failure the β_2-adrenergic receptors hinder the already diminished β_1-adrenergic receptors system from stimulating heart activity.

These findings are of interest because they indicate that a given GPCR must be able to generate different intracellular surfaces to attract such functionally and structurally distinct $G\alpha$ subunits. Furthermore, in the case of the β_2-adrenergic receptor, since $G\alpha_i$ and $G\alpha_s$ induce opposing actions, it raises the questions of how does the receptor know to stimulate or inhibit adenylyl cyclase and what molecular influences, such as agonists or other regulatory proteins, direct the receptor to act one way versus another.

The diversity of GPCR/G protein association has interesting implications with regards to drug discovery. Thus, if drugs can be identified to change the pattern of GPCR/G protein associations or cause a GPCR to preferentially associate with one G protein and not others, then it would be possible to shift the pharmacological profile and functions of a given receptor. This could be used to remove side effects associated with activation of a given receptor while maintaining desired therapeutic actions or produce new drug effects via a given GPCR. Since GPCR associations with individual G proteins would require a unique structural basis due to the conformation generated by agonist binding, then developing drugs that reproduce that given conformation could direct GPCRs to couple to selective G proteins. This may be accomplished either by finding drugs that bind in a unique manner to the ligand binding domain or by identifying allosteric regulators.

Allosteric regulators interact with GPCRs at sites topographically distinct from the classical ligand binding domains.[48–50] G proteins act upon allosteric sites to affect GPCR conformation, and drugs targeting these unique GPCR/G protein contact sites could prevent coupling of the receptor with some G proteins but not others. Such allosteric regulators could be specific for targeting GPCRs and produce selective therapeutic properties. For example, targeting somatostatin type II receptors in islet beta cells to couple to $G\alpha_q$ and not couple to $G\alpha_i$ could result in a receptor that stimulates Ca^{2+} mobilization to increase insulin release instead of a receptor that normally inhibits insulin release.[12] Such allosteric regulators of the somatostatin type II receptor could be used to treat diabetes.

Allosteric regulators are of interest because they can be highly selective for a given GPCR. They are also limited in their maximal effect on a receptor and therefore cannot excessively stimulate or inhibit the receptor as they only act in the presence of endogenous ligands or agonists.[51] Importantly, they can be used to modulate the maximal effectiveness of full agonists at that receptor, and in effect they can tone down the receptor activation. This can be useful in prolonging the activation of the receptor and diminishing desensitization. It will be of interest to determine whether such regulators can also be used to switch such dual-acting receptors as the β_2-adrenergic receptor from one that stimulates to one that inhibits adenylyl cyclase. Since the inhibitory effects are cardioprotective, there may be instances where allosteric regulators could have therapeutic value in diminishing cardiac damage due to excessive stimulation or in the case of early stages of heart failure block the inhibitory effects of β_2-adrenergic receptors to facilitate cardiac output.

Several allosteric regulators of GPCRs have already been identified,[51] and there is major interest in the pharmaceutical industry to develop a new generation of allosteric regulators. Recently, cinacalcet HCl (Sensipar®, Amgen, Thousand Oaks, CA), an allosteric regulator of the Ca^{2+} sensing GPCR, has been approved by the Food and Drug Administration to treat hypoparathyroidism. Furthermore, Caden Biosciences (Madison, WI) has developed technologies that allow for allosteric regulator drug discovery, by targeting drugs at the G protein binding sites of GPCRs. This suggests that allosteric drugs may be a large untapped field of discovery of novel GPCR therapeutics.

GPCRs and Arrestins

In addition to G proteins, GPCRs also couple with G protein receptor kinases (GRKs) and arrestins (also referred to as β-arrestins), which are involved in desensitization and termination of receptor activation following prolonged agonist binding.[40,52,53] There are four different β-arrestins and seven different GRKs in the human genome involved in regulating GPCRs.[40,52] Studies on the β-adrenergic receptor, as well as other GPCRs, have shown that following prolonged agonist binding to GPCRs, cytosolic GRKs are induced to translocate to GPCRs where they catalyze the phosphorylation of serine or threonine residues on the intracellular loops and C-terminal tail of the receptor (Fig. 1). This phosphorylation attracts β-arrestins to the receptors, which compete with G proteins for binding to the cytoplasmic side of the receptor. In effect, β-arrestins uncouple GPCRs from G proteins, terminating signal transduction via G protein-mediated pathways and causing desensitization of some functional responses of the receptor. Furthermore, β-arrestins serve as adaptor molecules and link GPCRs to clathrin in recycling vesicles to facilitate the internalization of GPCRs.[54–56] GPCRs then undergo ubiquitination, which can lead to their targeting to the proteosome and lysosomal compartments.[57] This results in GPCR down-regulation. The translocation of GRKs and β-arrestins to GPCRs is believed to be a universal response to agonist activation and is critical for the inactivation of GPCRs and the termination of neurotransmitter and hormone action.

The GRKs and β-arrestins also have also been shown to have *in vivo* physiological roles in mediating the func-

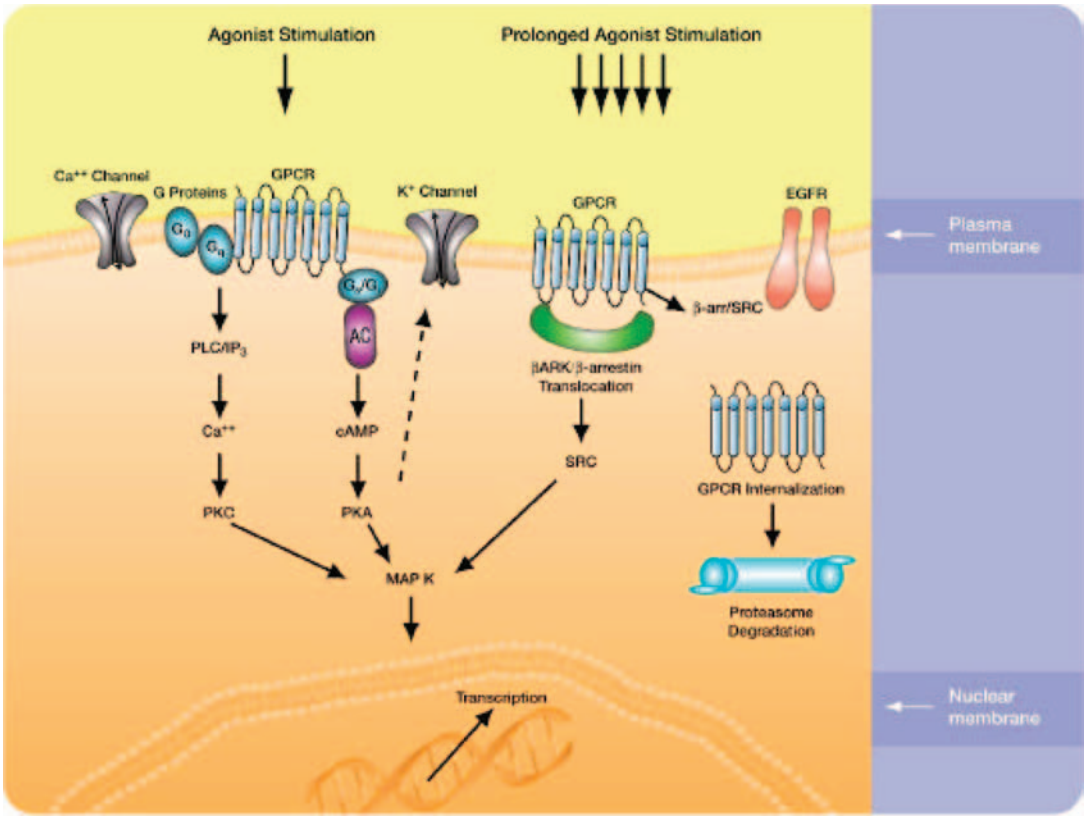

FIG. 1. Regulation of GPCR signaling by β-arrestins. (**Left side**) Stimulation of GPCRs in cells acutely by agonists leads to activation of multiple signaling pathways, including those involving second messengers such as cAMP, IP3, and Ca^{2+}. GPCRs in the plasma membrane couple to these pathways via G proteins that link the receptors to enzymes such as adenylyl cyclase (AC) and phospholipase C (PLC) or ionic conductance channels including the Ca^{2+} and K^+ channels. PKA, protein kinase A; PKC, protein kinase C. (**Right side**) Prolonged stimulation of the receptor leads to recruitment to the cell membrane of β-adrenergic receptor kinases (βARK) and β-arrestin. This leads to uncoupling of the GPCR from G proteins and the second messenger pathways and leads to three subsequent and parallel processes. β-Arrestin (β-arr) couples the GPCR to SRC, which can link the receptor to MAPK and to growth factor receptors such as the epidermal growth factor receptor (EGFR). This creates alternative signaling via the GPCR, in contrast to its G protein-mediated functions. β-Arrestin also serves as an adaptor linking the receptor to clathrin-coated vesicles, which internalize the receptor. This can lead to targeting the receptor to degradation in the proteosome. Adapted from Lefkowitz and Shenoy[40] and Shenoy and Lefkowitz.[54]

tions of GPCRs. Thus, the knockout of β-arrestin2 in mice leads to enhance analgesia induced by morphine and opiates and greatly diminished tolerance to these drugs.[58,59] Tolerance is an *in vivo* correlate of desensitization and is a major therapeutic limiting side effect of opiates. Interesting, opiate tolerance occurred in mice lacking β-arrestin1, suggesting selectivity in the interaction of different β-arrestins with GPCRs. Furthermore, in mice lacking β-arrestin2, while opiate-induced tolerance was diminished, opiate dependence was not reduced. This suggests that dependence does not require tolerance to occur and therefore is likely to be mediated via distinct molecular mechanisms.

GRKs and β-arrestins act as safety mechanisms to prevent the overstimulation of GPCRs. While the translocation and association of β-arrestins to GPCRs are relatively rapid, they are still much slower than typical GPCR activation of G proteins and intracellular signaling pathways. The uncoupling of the receptors from G proteins and GPCR internalization is sequentially much slower than the processes initiated by acute GPCR stimulation. Thus, the β-arrestins provide a dampening mechanism to preserve GPCR sensitivity to endogenous ligands to maintain the quick responses evoked by neurotransmitters and hormones.

The importance of GRKs and β-arrestins in mediating GPCR desensitization suggests that these molecules could be important targets for the development of drugs to prevent tolerance development to established drugs and prolong the therapeutic activity of these agents. Some degree of specificity exists with regards to which GRKs or β-arrestins interact with which GPCRs. Thus, in the case of morphine's actions, tolerance was diminished in animals lacking β-arrestin2 but not β-arrestin1. Further, mice lacking GRK5 display enhanced responses to muscarinic agents, while those lacking GRK6 have enhanced responses to dopaminergic agonists.[21,60] Importantly, as described in more detail below, assays are now available to measure translocation of β-arrestin to GPCRs following agonist stimulation. These assays can be employed for drug screening. Thus, if individual β-arrestins interact with unique recognition sites on the intracellular domains of selective populations of GPCRs, then it may be possible to identify compounds that selectively block the desensitization of some receptors but not others to prolong the actions of some therapeutically relevant drugs. For example, β-arrestin-targeted drugs could be useful in diminishing tolerance development to morphine to allow more prolonged analgesia in patients with chronic pain.

β-Arrestins, however, do not just simply act to turn off GPCR functions. In fact, more recent evidence suggests that they act as switching mechanisms to convert GPCR function from one dependent on G proteins to one independent of G proteins.[40] Thus, β-arrestins can function as adaptor molecules to recruit c-Src to agonist-bound GPCRs (Fig. 1). c-Src can in turn cause the phosphorylation of critical tyrosine residues on the epidermal growth factor receptor. Thus, β-arrestins provide a means of cross-talk between GPCRs and growth factor receptors.

Furthermore, β-arrestins can link GPCRs to the MAPK pathway. G proteins can also link GPCRs to the MAPK system, but via different mechanisms and with different cellular consequences.[61] Thus, G proteins link GPCRs to protein kinase C and A, which phosphorylate and active the multicomponent MAPK pathway to phosphorylate transcription factors in the nucleus. This effect is rapid and short-lived and involves members of the MAPK pathway translocating to the nucleus to phosphorylate transcription factors.[40] In contrast, β-arrestins act as a scaffold to recruit c-Src and other members of the MAPK pathway to GPCRs. The β-arrestin activation of the MAPK pathway, in contrast to the G protein-mediated pathway, is slower in onset and more prolonged and does not appear to primarily involve phosphorylation of nuclear transcription factors.[40] This process appears to predominantly regulate cell motility, chemotaxis, and apoptotic processes in cells.

Thus, in effect, when GPCR activation causes translocation of β-arrestins to the cell membrane, G protein signaling is terminated, and GPCRs are targeted to endocytic vesicles where they are internalized either to be degraded or eventually to be returned to the cell surface (Fig. 1). While GPCR signaling via G proteins is lost, GPCR signaling via β-arrestins is initiated, leading to activation of growth factor signaling pathways and stimulation of c-Src and MAPK pathways causing long-term changes in cell activity distinct from those resulting from acute stimulation of GPCRs.

The duality of GPCR responses via G protein versus β-arrestins mechanisms may provide interesting possibilities for novel drug discovery. Thus, as mentioned above, drugs targeting the inhibition of GPCR/β-arrestin associations could be useful in prolonging agonist activation of the GPCR/G protein pathway. Azzi *et al*.[62] have reported that inverse agonists at the β-adrenergic receptor, while reducing cAMP basal levels via a G protein mechanism, increased MAPK activity via a mechanism independent of G protein but instead mediated by β-arrestins. Thus, ligands acting via the same receptor can produce distinct cellular responses via either G protein or arrestin pathways. Employing assay systems that can simultaneously measure responses via the G protein pathway (changes in cAMP or IP_3 levels) and β-arrestin pathway (translocation of β-arrestins to GPCRs) could provide the means to design and develop unique drugs targeting one pathway or the other.

GPCR Oligomerization

While GPCRs are generally considered as monomeric proteins, there is growing evidence to suggest that GPCRs can exist as oligomers.[63–67] Importantly, if hetero-oligomers bind ligands differently than homo-oligomers, then the possibility exists to develop drugs that distinguish oligomers that could provide distinct selectivity and therapeutic value not seen in classical drug discovery efforts (Fig. 2).

Oligomerization has been known for years to be essential for signal transduction via cell surface receptors, in particular for growth factor receptors.[68–73] In fact, essentially every growth factor receptor consists of either homo- or hetero-oligomers. Oligomerization occurs when extracellular growth factors bind to one subunit that recruits other subunits. In some cases the subunit that binds the growth factor does not have inherent kinase activity, and the oligomerization is essential for instilling kinase activity in the receptor complex. In other cases the growth factor recognition subunit has kinase activity, and growth factor binding recruits other subunits that are substrates for the kinase and are essential for intracellular signaling to occur.

Oligomerization of neurotransmitter receptors is also known to exist for receptors containing ionic conductance channels.[34,74] The oligomerization is essential for creating the ionic conductance pore. Variations in the oligomer composition through the generation of hetero-oligomers are necessary for recruiting the neurotransmitter or hormone recognition sites to the channel as well as sites involved in second messenger regulation. Thus, there is

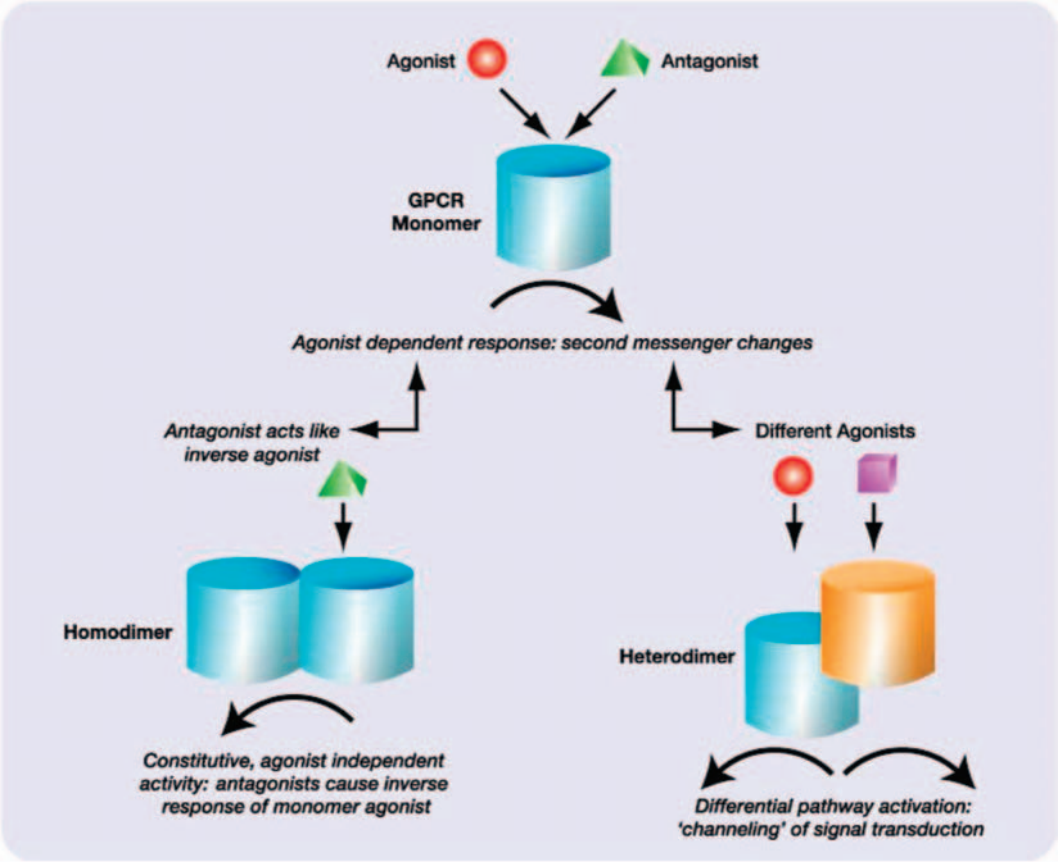

FIG. 2. GPCR oligomerization. The classical view of GPCR pharmacology suggests that receptors exist in cell membranes as monomers (**top**). Agonists bind to the receptor to stimulate signaling pathways in the cell, and antagonists bind the receptor, block the effect of agonists, but produce no effect of their own. More modern views of GPCR pharmacology suggest that GPCRs exist as oligomers, as either homodimers or heterodimers with other GPCRs (**bottom**). With homodimers (**bottom left**), the GPCRs can express constitutive activity, functional responses in the absence of activating agonist. Antagonists can bind to the receptor, block effects of agonists, but also induce by themselves inverse agonism, which comprise responses that are opposite to those induced by agonists. For heteromers (**bottom right**), because two different receptors are associated in one complex and the ligand binding regions of the two receptor still recognize selective ligands, the possibility exists that the complex can take on the pharmacology of the two different receptor families, or new pharmacology is created by the fusing of the different ligand binding domains. This can result not only in variations in ligand recognition, but also in the heteromer coupling to multiple signaling pathways.

considerable evidence for the role of oligomers in signaling via integral cell membrane proteins.

The first suggestions that GPCRs may form oligomers occurred when prototypical receptors such as the β-adrenergic receptor were characterized using biochemical approaches such as radiation inactivation. The molecular weight estimates based on this approach were very different from those used to determine the molecular weight of the purified receptors in a monomer form.[5,9] While these discrepancies could be due to a number of factors, one explanation for the differences was the formation of dimers of the receptors. Additional evidence that GPCRs form oligomers came from co-immunoprecipitation studies of GPCR complexes using antibodies directed against individual receptors in the oligomer.[75] In fact, GPCRs that respond to hormones such as gonadotropin-releasing hormone form disulfide bridges in their N-terminus and exist as dimers. Such biochemical studies have indicated that some GPCRs primarily form homo-oligomers.

Substantial evidence exists that homodimers can form constitutively active GPCRs that are receptors that are active without the presence of ligand[76–87] (Fig. 2). Homodimers can form either by mutations in the GPCR or when receptors are overexpressed. This latter point is important because most drug screening assays employing recombinant GPCRs use cell lines overexpressing the receptors, and the expressed receptors may be a mixed population of monomers and oligomers, with the latter being constitutively active. The partial constitutive activity may explain the elevated basal responses such as cAMP or IP3 formation seen in cell lines expressing high levels of recombinant receptor. As a consequence, many of the drugs identified in such screening studies may primarily act on homodimers and may or may not act on monomeric receptors. This can be a problem since it is likely that most GPCRs naturally expressed in the body at relatively low levels are monomeric. Consequently, drugs identified by their interaction with homodimers in drug screening studies may not be effective *in vivo* or produce different effects *in vivo* than *in vitro*.

Because constitutive activity generally raises basal second messenger signaling, homodimer drug screening may be particularly useful in developing inverse agonists, which by definition produce opposite effects compared with classical agonists (Fig. 3). Importantly, inverse agonists interact with the activated state of GPCRs and reduce basal activity of the activated receptor (Figs. 2 and 3). In contrast, classical agonists bind to the inactive receptor and stimulate the receptor to increase activity. Thus, the two sets of compounds produce opposing actions through distinct mechanisms via the same receptor (Fig. 2).

There is tremendous interest in the pharmaceutical industry in developing inverse agonists, which may have therapeutic advantages over classical agonists. In addition, practical advantages of inverse agonists are their potential use in treating disorders caused by constitutively active GPCRs. Interestingly, studies on a mutant β_2-adrenergic receptor have shown that the receptor became constitutively active with regard to regulation of the Na^+/H^+ ion exchanger, one of the cellular effector responses to adrenergic stimulation.[87] However, the same mutant receptor did not have a modified basal adenylyl cyclase activity. This finding suggests that constitutive activity may not necessarily encompass all functions of the GPCR and also reinforces the idea that GPCRs couple to different effector systems through distinct mechanisms.

GPCRs are also able to form hetero-oligomers (Fig. 2). This is the case for the dopamine D1 and D2, sero-

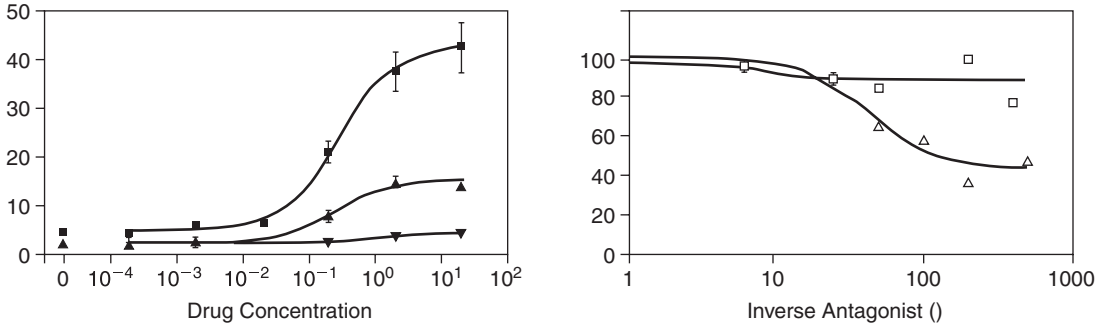

FIG. 3. GPCR agonism and inverse agonism. (**Left**) Classical GPCR pharmacology has shown that full agonists (■) produce maximal activation of the receptor, as shown on the dose–response curve. Partial agonists (▲) are compounds that have a mixture of agonist and antagonist properties because they can produce less of the maximal response than the full agonist and when added to full agonists can reduce the potency of the full agonist to activate the receptor. Neutral antagonists (▼) produce no response of their own but block the effects of agonists. (**Right**) With oligomeric GPCRs, most antagonists (△) can bind to the receptors and produce inverse agonism. This results in a decrease in basal activity (□) and responses opposite to those seen with full agonists.

tonin 5-HT1B, the angiotensin 1 (AT1) and B2 bradykinin receptors, and the δ- and μ-opioid and CCR5 receptors (Family 1), the gonadotropin-releasing hormone receptor (Family 2), and the γ-aminobutyric acid $GABA_{B1}$ and $GABA_{B2}$ and metabotropic glutamate receptor mGluR1 receptors (Family 3). Importantly, each of these receptors is known to form homomers as well as GPCR heteromers.[65,67,88,89]

A number of studies have suggested that heterodimers may have critical functional properties distinct from homodimers. This was first suggested in studies on the metabotrophic $GABA_B$ receptors. The $GABA_{B1}$ receptor when expressed alone does not reach the cell surface and is functionally inactive. The $GABA_{B2}$ receptor when expressed alone in cells reaches the cell surface but is also functionally inactive because it does not bind ligand.[90] However, when the $GABA_{B1}$ and $GABA_{B2}$ receptors are co-expressed as heterodimers, they translocate together from the endoplasmic reticulum to the cell surface, and the $GABA_A$ receptor then becomes functionally active.[91,92] Studies by Margeta-Mitrovic *et al*.[91] showed that the $GABA_{B1}$ has a sequence that causes it to be retained in the cytosol. When the receptor associates with the $GABA_{B2}$ receptor, this retaining region of the receptor is masked so that both receptors can be sorted to the cell surface.

Importantly, the two receptors have different functions and only after oligomerization is a fully functional receptor reconstituted. Thus, the $GABA_{B1}$ receptor has ligand binding properties, but does not couple to G protein and adenylyl cyclase, whereas the $GABA_{B2}$ receptor couples to G proteins and adenylyl cyclase but cannot bind GABA.[90,93–95] Thus, hetero-oligomerization is essential for mediating effects of GABA via metabotrophic receptors.

Furthermore, oligomer formation has been suggested to change the G protein coupling and signaling pathways of GPCRs. Breit *et al*.[96] showed that β_2/β_3-adrenergic receptor heteromers had different G protein coupling than the homomers, and Mellado *et al.*[97] found that CCR2 and CCR5 chemokine heteromers had different G protein coupling than the homodimers.

Interestingly, Lee *et al.*[98] found that heteromers of the dopamine D1 and D2 receptors are expressed in brain and respond to agonist stimulation with increases in intracellular Ca^{2+}, whereas stimulation of either D1 or D2 receptor homomers does not. The heteromer acts via a phospholipase C/G_q pathway to mediate this response, whereas the monomers do not associate with G_q. These findings are of interest because interactions of D1 and D2 receptors have been reported in behavioral and electrophysiological studies, and hetero-oligomer formation of the receptors may be the molecular basis of that interaction. Importantly, interactions of these two receptors may become dysfunctional in a number of central nervous system disorders including schizophrenia and Parkinson's and Huntington's diseases, as well as other mental disorders. Thus, drugs that may modulate D1/D2 receptor oligomerization and either maintain or disrupt such interaction could be therapeutically important in treating these diseases.

Cross-talk has been reported for a number of other GPCRs[63,67] and may be a common phenomenon in the function of GPCR oligomers. These studies suggest the cross-talk may involve differential coupling of the heteromers to G proteins compared to either the homomers or monomers. This could indicate that depending on the form of the GPCR, the functions may differ.

Hetero-oligomers also appear to be regulated differently than homo-oligomers. So *et al.*[99] found that D1/D2 heteromers were regulated differently than homomers. Dopamine D1-selective agonists and D2-selective agonists were able to cause internalization of the heteromer, and D1-selective agonists were able to induce phosphorylation of the D2 receptor. In contrast, D2-selective agonists did not cause internalization of D2 homomers. Breit *et al.*[96] showed that hetero-oligomers of the β_2- and β_3-adrenergic receptors have similar ligand binding properties as the homo-oligomers, but β_2-adrenergic agonists do not cause internalization of the hetero-oligomer, and the ability of agonists to cause translocation of β-arrestin to the heteromer is reduced compared to the β_2-receptor homomer. This suggests that β_2/β_3-receptor heteromers may be regulated differently than β_2-receptor homomers and are less likely to desensitize.

In addition to changing the G protein coupling and signaling of GPCRs and regulation of the receptors, hetero-oligomer formation may also change ligand binding properties of the receptors. Hilairet *et al*.[100] showed that cannabinoid receptor CB1 and the orexin 1 receptors form hetero-oligomers. They found that orexin was 100-fold more potent in stimulating CB1/orexin 1 hetero-oligomer than orexin 1 homo-oligomers. This study was particularly important because it provides a direct basis for the interaction of the cannabinoid and orexin systems and suggests that CB1 antagonists, which are being proposed as a treatment of obesity, may do so by blocking both the actions of endogenous cannabinoids and the orexin system.

The pharmacological properties of other GPCRs have also been suggested to change when they form hetero-oligomers. George *et al*.[101] reported that κ- and δ-opioid receptors form hetero-oligomers with reduced affinity for either κ- or δ-opioid-selective ligands, whereas compounds that are nonselective at these opioid receptor subtypes had greatly enhanced potency at the hetero-oligomer compared to the homomers. These findings suggest that the ligand binding pocket of the heteromer may differ from that of the homomer. If this occurs, then it suggests that it may be possible to discover drugs that selectively bind to the heteromer.

Developing compounds that selectively bind to heteromers could result in drugs with unique and potentially beneficial therapeutic properties.[102] For example, most of the clinically employed opioids used to treat pain also produce significant side effects such as respiratory depression and nausea.[14] These drugs primarily act upon μ-opioid receptors to cause these side effects. The analgesic properties of opiates are primarily mediated by μ receptors in the thalamus and spinal cord, whereas many of the side effects of the drugs are due to actions of the opiates in brainstem nuclei. The μ receptors are known to form hetero-oligomers with a number of different GPCRs, including the κ and δ receptors. The κ and δ receptors are expressed in thalamus and spinal cord, where they mediate analgesic actions of dynorphin and enkephalin, but they are not expressed in the brainstem, and κ- and δ-selective agonists do not cause respiratory depression or nausea. Thus, if agonists could be developed that selectively activate either μ/κ or μ/δ receptors but not μ receptor homomers, then it may be possible to generate analgesics without many of the major side effects of clinically used opioids.

However, some investigators have suggested caution with regard to the importance of GPCR oligomers. First, most evidence regarding oligomers has been found with recombinant receptors overexpressed in cell lines. Those expression levels are rarely seen in tissues in the body, and therefore the formation of native oligomers may be infrequent *in vivo*. Second, James *et al*.[103] recently employed bioluminescence resonance energy transfer (BRET), a technology commonly used to identify oligomers, to study GPCR homomer and heteromer formation, and they suggested that some of the heteromer formation reported by others for GPCRs, including those for β_2-adrenergic and cannabinoid receptors, may be a result of "random collisions" rather than specific dimer formation. The implication of their work is that the technologies used to examine protein–protein interaction are so sensitive that nonfunctional random interactions between GPCRs would be detected just as well as functionally important oligomers. They employed several methods to distinguish specific oligomer formation, such as the formation that occurs between the $GABA_{B1}$ and $GABA_{B2}$, from nonspecific associations, such as the association that occurs with the β_2-adrenergic and cannabinoid receptors. As a consequence, they suggested that some of the oligomers identified using this approach by others are due to less than rigorous analysis of the BRET technology, and they implied that the occurrence of actual oligomers is rarer than first believed. Importantly, this suggests that multiple technologies, including analysis for physical as well as functional associations, are needed to authenticate true GPCR oligomers from those that appear as an artifact of the technologies employed.

Orphan GPCRs as Drug Discovery Targets

Bioinformatic analysis of the human genome has identified a large number of proteins as putative GPCRs based on structural similarities (predicted seven-transmembrane structure with consensus sequences such as the DRY amino acid sequence in transmembrane regions) to the 200 or so known GPCRs whose endogenous ligands and functions are known. These putative orphan GPCRs do not have known ligands, and in most cases their functions are not known. However, in many cases, information on their expression patterns and localization in the body has provided tantalizing evidence of their potential roles in physiology, and as a consequence much interest has been generated in the pharmaceutical industry in developing drugs against these targets as unique and effective agents to treat disease and other disorders.[104]

Because of this interest for drug discovery, attempts have been made to de-orphanize these receptors.[2,3,105,106] De-orphaninzation can involve multiple approaches and technologies. The one commonly employed was first described by Stadel *et al.*[104] and can involve developing assay systems to measure responses of these receptors when stimulated and then employing laborious procedures to extract endogenous ligands from tissues and testing such extracts for activity in screening systems or simply screening the assays with small molecule, peptide, or natural product libraries to identify agonists.

This approach is not new; in fact, it was employed for the identification of many GPCR endogenous ligands over the years, notably the endogenous opiates in the 1960s and 1970s. Interestingly, one of the first successes in de-orphanizing GPCRs involved ORL-1, a receptor whose sequence was identified in the initial efforts to clone the δ-, κ-, and μ-opioid receptors. ORL-1 has approximately 65% amino acid sequence similarity to these opiate receptors. However, known opiate ligands did not bind effectively to this receptor. Like opiate receptors, ORL-1 is linked to G proteins and mediates inhibition of adenylyl cyclase. Using a cell-based cAMP assay to identify stimulators of this receptor, both Meunier *et al*.[107] and Reinscheid *et al.*[108] were able to extract from brain an endogenous peptide, orphanin FQ or nociceptin, as an agonist at the receptor. ORL-1 has generated much interest in the pharmaceutical industry because nociceptin ligands may have important clinical advantages over opiates since they can produce analgesia, but in contrast to opiates they are not known to induce physical dependence and other serious side effects that limit the clinical use of traditional opiates. Importantly, ORL-1-targeted drugs may be particularly useful in treating neuropathic pain due to peripheral nerve injury, and presently there are no drugs effective in treating this disorder.

Following the identification of endogenous ligands at the ORL-1 receptor, further efforts were made by phar-

maceutical companies to employ similar strategies to identify endogenous ligands and to develop drugs against other orphan GPCRs. Screening brain or stomach extracts for endogenous ligands led to de-orphanization of orexin, ghrelin (growth hormone secretagogue receptor), prolactin releasing peptide (GPR10), apelin (APJ), neuropeptide B&W (GPR7&8), and urotensin II (GPR14) receptors. The orexin receptors have garnered much interest because of their role in sleep, eating, and addictive behaviors, and antagonists targeting those receptors have potential for treating a number of disorders related to those behaviors. In fact, mutations in the orexin-2 receptor have been shown to be involved in canine narcolepsy and mild sleep disorders in humans.

Identification of urotensin II as the endogenous ligand for the orphan GPR14[109] was of particular interest since this peptide is the most potent vasoconstrictor known, and the receptor became an important target for a major effort to discover novel antihypertensive agents. Finally, studies at Merck showing that the orphan GPCR HG55 was responsive to cysteinyl leukotrienes[110] have led to a major interest in developing drugs targeting this receptor as novel anti-inflammatory and anti-asthma therapeutics.

These successes in de-orphanization and identification of novel endogenous ligands have led to a major emphasis on drug discovery and development targeting orphan GPCRs. This, together with the idea that some recently de-orphanized GPCRs, such as orexin receptor, may dimerize or associate with more classical GPCRs, suggests that there is abundant room for drug discovery against both the orphans themselves and the systems they co-regulate or influence.

GPCR Mutations, Disease, and Novel Drug Discovery

While GPCRs are critical for mediating the normal functions of neurotransmitters and hormones, they also play a role in a number of diseases. Loss-of-function mutations in GPCRs involved in the control of endocrine systems, such as the receptors for adrenocorticotropic hormone, follicle-stimulating hormone, gonadotropin-releasing hormone, growth hormone-releasing hormone, thyrotropin-releasing hormone, and thyrotropin (thyroid-stimulating hormone), mimic the symptoms found when those hormones are not expressed appropriately.[85,111,112] For example, loss-of-function mutation of the thyroid-stimulating hormone receptor causes congenital hypothyroidism.[113] Mutations of the endothelin type B receptor can result in Hirschsprung's disease, a congenital disorder involving bowel obstruction,[114] and loss of function of parathyroid hormone receptors causes Blomstrand's chondrodysplasia, a disorder associated with abnormal breast and bone development.[115] Furthermore, homozygous loss-of-function mutations in the type 5 chemokine receptor provide resistance to human immunodeficiency virus infection because this receptor is critical for the infectivity of this virus.[116] While explaining how some individuals who have been exposed to the virus do not get the disease, it has also spurred interest to develop antagonists against this receptor as a potential treatment of human immunodeficiency virus infection.

Loss-of-function mutations in general are difficult to develop treatment strategies for, since if the receptor is not functional, then how does one develop drugs to repair its activity? However, one of the more interesting developments of disease treatment occurred with the vasopressin V2 receptor. Loss-of-function mutation in the vasopressin V2 receptor causes X-linked nephrogenic diabetes insipidus.[117] Many of the mutations appear to prevent appropriate sorting of the receptor to the cell surface. thereby preventing the actions of vasopressin in water absorption. As a consequence the receptor either remains in the endoplasmic reticulum or gets sorted to intracellular sites for degradation. Morello *et al*.[118] and Bernier *et al*.[119] found that small molecule antagonists of the V2 receptor that are cell permeable were able to rescue the mutant V2 receptors and allow for surface expression and function of the receptors; peptide antagonists that do not enter the cell did not rescue the receptor function. Furthermore, clinical trials with cell-permeable V2 antagonists were able to promote water absorption in patients with nephrogenic diabetes insipidus, suggesting this approach may be a viable route to treat this disease.

The V2 receptor is not the only one where pharmacological chaperones proved effective in treating a GPCR disease. Janovick *et al*.[120] reported that mutations of the gonadotropin-releasing hormone receptor also cause inappropriate sorting of the receptor to the cell surface. They found that small molecule antagonists of this receptor allowed for surface expression of the mutant receptors.[121] Such drugs now become a potential treatment for hypogonadism. Thus, while it may not be easy to develop drugs to compensate for loss-of-function mutations in GPCRs in which the mutation prevents either agonist binding or activation of the receptor, these studies suggest that drugs may be identified as chemical chaperones that are able to bind mutant receptors and relieve defective sorting or processing to treat the disease.

The use of chemical chaperones to treat disease is not limited to GPCRs. In cystic fibrosis, there are mutations in the cystic fibrosis chloride channel (CFTR) that diminish channel functioning to cause lung pathology. Close to 1,000 mutations have been identified in CFTR, but the one most prominent and most extensively studied is ΔF508.[122] Over 90% of patients with cystic fibrosis have this mutation. The mutation is believed to cause a misfolding of the channel so that it does not effectively get transported from the endoplasmic reticulum to the cell

surface and causes it to have a shorter half-life than the wild-type channel because the abnormal protein is recognized by the proteasome, which more rapidly degrades CFTR ΔF508 than the wild-type channel.[122] If the mutant channel gets to the cell surface, it is functional and able to mediate Cl^- efflux and conductance, although at a diminished level compared to the wild-type channel. Pharmacological studies have suggested that CFTR ΔF508 function can be partially rescued, by treatment with small molecules such as trimethylamine-*N*-oxide, which are believed to stabilize misfolded proteins and increase surface expression and functioning of CFTR ΔF508 *in vitro.* Trimethylamine-*N*-oxide was also effective in increasing the mutant channel activity in transgenic mice expressing CFTR ΔF508. This has led to the proposal that compounds such as trimethylamine-*N*-oxide may serve as chemical chaperones to the mutant channel to treat cystic fibrosis by increasing trafficking and functioning of the mutant CFTR,[122] much like GPCR antagonists may be useful in treating diseases caused by diminished receptor trafficking.

In fact, development of pharmacological chaperones may have a wider role in treating a number of diseases associated with protein misfolding and inappropriate sorting such as Alzheimer's, Parkinson's, and Huntington diseases as well as many other diseases of the nervous system and other systems in the body. Since many of these protein-sorting disorders are heterozygous, the question arises in the case of GPCRs whether drugs that selectively interact with mutant receptors can be identified to rectify the defect but not alter the normal functioning of wild-type receptors. Such drugs might be expected to have high selectivity in treating the disease, since they would only bind to mutant receptor and not the wild-type receptor.

In addition to loss-of-function mutations, gain-of-function mutations in GPCRs also cause disease. In most cases the gain of function is related to conversion of the wild-type receptor that is dependent on agonist stimulation to a constitutively active receptor that is not dependent on activating ligand. Gain-of-function disorders include constitutively active rhodopsin, which can cause night blindness,[123] constitutively active parathyroid hormone-related receptor, which causes Jansen-type metaphyseal chondrodysphsin,[124] constitutively active thyroid-stimulating hormone and follicle-stimulating hormone receptors, causing congenital hyperthyroidism[125] and familial male precocious puberty,[126] and mutations in the calcium sensing GPCR that cause hypocalciuric hypercalcemia and neonatal hyperparathyroidism.[127] For these diseases, development of inverse agonists could be useful as therapeutics since they would be predicted to selectively block the actions of the constitutively active receptor. These would be especially important if drugs could be developed to target the oligomers and not interact with monomeric receptors so that the drugs would not affect the normal functioning of the GPCRs in the body.

While determining phenotypes of GPCR mutations that cause dramatic changes in activity has been possible, more subtle biological consequences of most mutations or polymorphisms have been more difficult to determine. For example, a well-known polymorphism in the β_3-adrenergic receptor occurs in populations of American Indians and has been linked to weight gain and early-onset non–insulin-dependent diabetes.[128] This mutation is known to cause a decrease in basal and agonist-stimulated adenylyl cyclase activity via this receptor.[129] This mutation is of interest because the β_3-adrenergic receptor is involved in fat storage in adipocytes, and altered signaling via this receptor might be expected to cause changes in metabolism. However, it is controversial whether reduced functioning of this receptor is really related to obesity and predisposition to Type II diabetes. Indeed, polymorphisms have been identified for many amine receptors including adrenergic receptors that have been linked to congestive heart disease,[130] and mutations in a number of dopamine and serotonin receptors have been linked to central nervous system disorders such as schizophrenia, Parkinson's disease, and attention deficit hyperactivity disorder. However, a direct cause and effect link of these mutations with disease is still not clear.

While direct cause and effect links have not been made, it is possible that mutations in GPCRs could be responsible for variations in drug sensitivities among different populations. Such mutations might not only cause variations in effectiveness or potency of GPCR-directed drugs, but also drugs that are indirectly active via GPCRs such as neurotransmitter uptake inhibitors, including those used to treat depression, since changes in sensitivity could determine how the endogenous transmitters activate GPCRs as well as drugs. The influence of GPCR polymorphism on how drugs affect humans could potentially be very important, especially since we primarily view changes in drug sensitivities with regard to variations in metabolism via the P450 system and the P-glycoprotein pathway. Since there is now a greater and greater effort in pharmcogenetics to try to link the genetic makeup of an individual to both disease susceptibility and drug responsiveness, then mutation profiles in GPCRs should also be considered in such genetic evaluation especially since many of the therapeutics we use today are directed against GPCRs.

Importantly, if polymorphisms cause subtle changes in GPCR functioning, then the development and use of allosteric regulators and inverse agonists may come into play as important therapeutics. For example, if mutations in the β_3-adrenergic receptor are really responsible for the appearance of obesity and Type II diabetes and this is due to a subtle loss of activity via this receptor, then

allosteric regulators that diminish the desensitization of this receptor could serve to compensate for the loss of activity of the mutant receptor. Furthermore, if hyperactivity of dopamine receptors occurs in schizophrenia, which was the rationale for the development of the first generation of antipsychotic drugs, then inverse agonists that subtly tone down these receptors could be useful therapeutics potentially without the side effects of classical dopamine receptor antagonists. Clearly a better understanding of the role of GPCR mutations in disease and the consequences of those mutations on the functioning the receptors may be critical in the development of entirely new families of GPCR modulators as therapeutics.

GPCR Chaperones and Regulators of Surface Expression

The finding that the mutant forms of the V2 vasopressin receptor and gonadotropin-releasing hormone receptor are unable to reach the cell surface yet can be "rescued" by pharmacological agents suggests that the chemical chaperones have a role in treating pathologies in which GPCR sorting is defective. Indeed, substantial evidence exists for several GPCRs with which protein chaperones are involved in cell surface expression.

For example, the metabotropic Group 1 glutamate receptors employ chaperones for their surface expression and cellular distribution. The glutamate receptor mGluR5 is a $G_{q/11}$-linked receptor mediating glutamate-induced increases in IP3 and diacylglycerol production in neurons. This GPCR is also involved in regulating neuronal excitability via modulation of Ca^{2+} and K^+ channels.[131] Surface expression of mGluR5 is regulated by a family of intracellular proteins referred to as Homer.[132–136] Homer 1b/c complexes with mGluR5 and retains the receptor in the cytoplasm. In contrast, the intermediate early gene product Homer 1a enhances surface expression of mGluR5, allowing the receptor to mediate biological actions of glutamate. In effect, Homer 1a competes with Homer 1b/c for binding to mGluR5. Importantly, Homer 1a is an intermediate early gene product, and its expression is induced following prolonged activation of neurons. This results in increased surface expression and therefore activity of mGluR5 in neurons. Thus, Homer 1a acts as a chaperone for mGluR5 and mediates the activity-dependent enhancement of mGluR5 trafficking and activity in neurons.

Homer proteins also determine whether mGluR5 becomes constitutively active. Studies by Ango *et al*.[137] showed that Homer 1a facilitates surface expression of mGluR5 and that the receptor becomes active even in the absence of agonist. Similar results were found with mGluR1. Thus, in this case, the chaperone not only targets the receptor to the cell surface but also increases its expression sufficiently to take on constitutive properties, possibly by oligomerization.

Interestingly, the Homer proteins are not only critical for surface expression and activity, but also distribution of mGluR5 in different compartments of neurons.[132] Expression of Homer 1b/c in brain neurons leads to mGluR5 expression in neuronal soma and dendrites, while Homer 1a expression leads to mGluR5 expression in axons as well as dendrites and soma. This is important because it suggests that Homer 1a allows for targeting of mGluR5 to nerve terminals.

The GPCR, mGluR5, is involved in mediating several glutamate-induced behaviors. Studies in mice with mGluR5 deletion show an important role of this GPCR in drug abuse since mice lacking this receptor did not self-administer cocaine.[138] Similarly, studies using a selective mGluR5 antagonist showed a role of this receptor in dependence induced by a number of drugs of abuse, including nicotine and cocaine.[139] Since many of these addictive drugs induce intermediate early gene expression, it is likely that a common mechanism by which they induce dependence may be their ability to increase the expression of the chaperone Homer 1a to increase mGluR5 surface expression to facilitate long-term activation of neuronal pathways (such as those in the nucleus accumbens) involved in drug abuse.

A second family of chaperones involved GPCR surface expression is the receptor activity-modifying proteins (RAMPs).[140,141] This protein family, designated as RAMP1–3, is of interest because it not only affects surface expression of GPCRs, but also may regulate processing of the receptors to affect their ligand sensitivity and thus could serve as targets for discovery of drugs to modulate GPCR responsivity to endogenous transmitters and hormones as well as drugs. Most research on RAMPs has focused on the calcitonin-receptor-like receptor (CRLR) (Fig. 4). The sensitivity of this GPCR to CGRP or adrenomedullin varies depending on its association with the RAMPs. CRLR oligomerizes with RAMP1 to form a complex with high sensitivity for CGRP. In contrast, association of CRLR with RAMP2 or 3 results in a receptor with high affinity for adrenomedullin.[140] Studies by McLatchie *et al*.[142] and Fraser *et al*.[143] showed that RAMPs were important for transporting CRLR to the cell surface, acting as chaperones. However, these results have been disputed by Flahaut *et al*.[144] Studies by Fraser *et al*.[143] showed that CRLR was transported to the cell surface by RAMP1 as a fully glycosylated protein, whereas the CRLR transported by RAMP2 or 3 was expressed as an immaturely glycosylated protein. These findings are interpreted to suggest that RAMP may be involved in modulating the processing of CRLR and that those differences in glycosylation are responsible for differences in ligand selectivity at the surface expressed receptor.

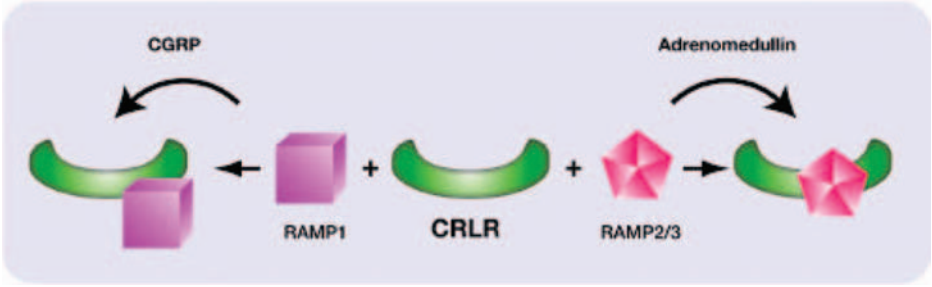

FIG. 4. RAMPs and GPCR functional diversity. RAMPs are cellular proteins that can associate with GPCRs and produce changes in the receptor to alter their ligand binding properties and pharmacological specificity. In the case of the CRLR, RAMP1 can associate with the receptor and generate a complex that recognizes CGRP (**left**). If RAMP2 or RAMP3 associates with CRLR instead, then a complex is generated that primarily recognizes adrenomedulin (**right**).

Differences in ligand selectivity may also be due to allosteric regulation of the GPCR by the RAMPs. Thus, RAMPs may induce conformational changes in CRLR to alter its specificity from a CGRP to adrenomedullin pharmacology. Furthermore, studies by Kuwasako *et al.*[145] suggested that RAMPs may contribute to the ligand binding pocket of CRLR to create variations in ligand sensitivity of the receptor. Consistent with this idea is the finding that RAMP1 contributes to the affinity of CRLR for small molecule CGRP antagonists.[146]

In addition to CRLR, RAMPs also mediate functions of other GPCRs. Thus, RAMP1 oligomerizes with the calcitonin receptor to generate a receptor with high affinity for CGRP and amylin. In contrast, association of RAMP3 with the same receptor generates a GPCR-selective for amylin. RAMPs interact with other Class II GPCRs, including those receptors for glucagons, parathyroid hormone, and vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide (VPAC).[141] For the VPAC receptors RAMPs appears to affect signaling via these receptors. Thus, the VPAC1 receptor is a $G\alpha_s$-coupled receptor that mediates stimulation of cAMP formation. When the receptor dimerizes with RAMP2 it is able to mediate agonist stimulation of IP3 formation as well as cAMP formation, suggesting that RAMP is able to expand the repertoire of G proteins with which that receptor associates.

The ability of RAMPs to modulate both ligand selectivity and G protein coupling of GPCRs suggests these proteins have important allosteric roles in regulating GPCR function. This also indicates that compounds targeting these proteins serve important pharmacological and therapeutic implications. Since the RAMPs have only 30% amino acid sequence identity,[142] enough structural difference exists between the proteins to provide the basis for development of selective drugs. Development of screening assays allowing for measurement of GPCR/RAMP interaction could provide the basis for the discovery and development of a novel family of drugs that serve as allosteric regulators of GPCRs.

GPCR Pharmacology

The fact that approximately 40% of the approved drugs target GPCRs and that there is an ever-growing list of GPCR drugs under development argue that approaches to discover GPCR drugs have historically been highly successful. These methods have advanced and become more sophisticated with the growing need to target more novel receptors and to discover drugs acting in unique ways to affect GPCR signaling. Classical drugs targeting GPCRs generally have fallen into two categories: agonists, which are drugs that mimic the actions of endogenous transmitters and hormones to stimulate GPCRs, and antagonists, which have no intrinsic activity of their own but which block activation of the GPCRs by agonists. This somewhat rigid pharmacological terminology has evolved over the years to embrace a wide spectrum of differently acting drugs.[147]

For example, agonists can be distinguished as full agonists, partial agonists, and inverse agonists, each with its own sets of advantage and disadvantages as therapeutics (Fig. 3). A full agonist is a drug that produces the same maximal effect as the endogenous neurotransmitter or hormone. Partial agonists are drugs that bind to GPCRs in a manner that produces less of an effect than full agonists. Partial agonists can antagonize full agonists. As a consequence, partial agonists exhibit duality in that they bind to GPCRs in a manner similar to both an agonist and an antagonist.

Partial agonists are therapeutically important because of their dual nature. For example, the μ-opiate receptor partial agonist buprenorphine is less effective than morphine in stimulating the μ-opiate receptor and antagonizes the actions of morphine at this receptor.[14] It was approved in 2002 by the Food and Drug Administration for treatment of opiate addiction because it blocks the actions of morphine and heroin at the μ-opiate receptor to allow for the addictive drugs to be tapered off while producing some stimulation itself, thereby preventing a full-blown withdrawal reaction.

While full and partial agonists have been known for many years, inverse agonists have been identified more recently in the last decade. Like partial agonists, inverse agonists are able to block the effects of full agonists at GPCRs. However, the unique property of these ligands is that they induce opposite effects on the same GPCR as full agonists (Fig. 3). Thus, whereas norepinephrine or isoproterenol will stimulate the β-adrenergic receptor to increase adenylyl cyclase activity, inverse agonists would bind to this receptor to decrease adenylyl cyclase activity. The inherent activity of an inverse agonist is dependent on the receptor having some level of constitutive basal activity.[147] In fact, as described above, most recombinant GPCRs overexpressed in cell lines produce constitutive basal activity that is caused in part by the generation of homodimers. Under these conditions, compounds that might otherwise be considered neutral antagonists produce inverse agonism. Inverse agonists may also be useful in pathological conditions where GPCRs undergo constitutive activity *in vivo* either because mutations cause the constitutive activity or because the receptors become overexpressed. Because elevated basal activity of a GPCR is needed to see inverse agonism, the question arises whether in tissues in which the endogenous receptor is expressed at low levels and basal activity is low do inverse agonists act on native receptors as inverse agonists or simply as neutral antagonists.

Technologies for GPCR Screening

Ligand binding assays

The approaches used to discover drugs against GPCRs involve either biochemical approaches, such as ligand binding assays using cell membrane preparations, or cell-based technologies. Ligand binding technologies, first developed in the early 1970s, were one of the linchpins in the GPCR drug discovery process.[148] Indeed, many Food and Drug Administration-approved GPCR drugs were first discovered using ligand binding technologies. The technique often requires the use of radioactive ligands that bind selectively and with high affinity to a given GPCR. The radioactive label enables detection of the ligand bound to the receptor in broken cell preparations, and a high affinity is required such that the rate of dissociation of the ligand from the receptor is relatively slow and the binding of the ligand to the receptor is sustained during washing procedures that eliminate nonspecifically bound ligand.

Many radioligands for GPCRs are now commercially available, including both agonists and antagonists. In general, these ligands are tagged either with tritium (^{3}H) or iodine-125 (^{125}I). A significant advantage of using of ^{125}I-labeled ligands is the very high specific activity that can be achieved, which can greatly increases assay sensitivity of the binding assay in comparison to assays employing ^{3}H-ligands. Consequently, these former assays detect lower levels of receptor, and therefore less tissue is needed for the assay. Nonetheless, ^{125}I-labeled ligands can cause concerns of equipment contamination due to the higher radioactivity. In addition, the half-life of the ^{125}I-labeled ligands is much shorter than those for ligands with ^{3}H; consequently, these latter ligands need to be used rapidly before extensive radioactivity decay.

In most receptor binding assays, consistent results are most often found using radioligands that are derived from antagonists, principally as the binding affinity is unaltered by changes in G protein association or other cellular factors, including ions that modulate radiolabeled agonist binding. However, this approach is not generally possible for many peptide receptors, many of which lack high-affinity antagonists. Most radioligand binding assays are easily adapted for screening and have the major advantage of providing clear evidence of direct interaction of a compound with the GPCR, an attribute that most cell-based assays lack.

There are, however, several disadvantages to the use of radioligand binding assays in screening. First, it requires the availability of a high-affinity, selective ligand that can be chemically radiolabeled. Consequently, this approach cannot be employed to study orphan GPCRs, which by definition lack known ligands. Second, even for many known GPCRs, the ligands available are of insufficiently high affinity to be used in classical receptor binding assays. Third, for many peptide receptors, the ligands available are peptides that generally need to be iodinated as radioligands. This requires extensive validation studies to optimize procedures to identify the best site in the peptide for insertion of the ^{125}I isotope such that the iodination does not affect the peptide pharmacology.

However, one of the most critical factors in restricting the use of radioligand binding assays for GPCR screening is the cost of radioactive waste disposal, particularly when large amounts are used in screening campaigns. Consequently, several nonradioactive alternatives have been developed for receptor binding assays using fluorescence detection.[149] However, such nonradioactive approaches have not been widely adopted in drug screening. This is due to the chemical complexity of attaching fluorescent groups to the ligand (without affect ligand/receptor pharmacology) as well as the relative insensitivity of the assay compared to radioligand techniques. Furthermore, it appears that the technology is primarily applicable to peptide ligands, thereby excluding many GPCRs to be studied.

Collectively, GPCR ligand binding technologies are employed to screen for drugs against any GPCR for which high-affinity ligands are available. They can be

used to distinguish agonist from antagonist binding. Antagonist binding is independent of G protein association with the receptor, and therefore binding is unaffected by GTP, which reduces G protein coupling, thereby converting GPCRs to a low-affinity state for agonist binding. Generally, agonist binding is detected by its sensitivity to a stable analog of GTP, namely, guanosine 5′-(γ-thio)triphosphate (GTPγS). However, this varies according to which G protein is associated with the receptor as well as the rate of GDP/GTP exchange for that G protein. Since GTPγS and sodium ions modulate agonist binding, the approach has also been employed to discover allosteric regulators of GPCRs.

A final point is that radioligand binding technology is readily adaptable to the study of native as well as recombinant GPCRs expressed either in membrane extracts or in intact cells. Indeed, it is one of the few screening approaches that can clearly define whether a ligand directly interacts with a GPCR. As a result, it has been has been widely used for identifying novel GPCR drugs as well as for profiling novel and known compounds.

Assays to measure GPCR/G protein association

Ligand binding assays indicate if a compound binds to a GPCR, yet provide relatively little information on the intrinsic activity of the compound. Since a functional consequence of the activation of GPCRs is the association with Gα and initiation of the binding of GTP to the α subunit followed by subsequent hydrolysis to GDP, screening assays that measure GPCR/G protein interaction have also been employed for drug discovery. One can determine GTPase activity as a measure agonist binding to GPCRs, and this assay was used extensively for research purposes in studying the kinetics of GPCR/G protein interaction. Simpler approaches used for HTS drug discovery involve measuring the ability of agonist to promote the binding of radiolabeled nonhydrolyzable GTP analogs, such as [^{35}S]GTPγS, as a measure of GPCR activation.[150] Such binding can be detected using the scintillation proximity assay format and is easily adaptable for HTS.[151,152] Furthermore, nonradioactive alternatives exist to measure GTPγS binding such as the DELFIA® Eu-GTPγS (PerkinElmer, Waltham, MA). These nonisotopic ligands have also been adapted for HTS with the advantage of reduced cost due to a lack of radioactive waste.

Generally, the advantages of this approach are that it can be used to detect agonist interaction with many GPCRs (excluding those GPCRs coupling to G proteins with low GDP/GTP exchange, such as $G\alpha_q$), including orphans for which little is known in terms of their second messenger coupling. The assay format can identify full agonists, partial agonists, and inverse agonists provided the basal activity is sufficiently high. Furthermore, it does not require the use of high-affinity agonists, only those that possess reasonably high efficacy. The approach can be also be employed to identify allosteric regulators of GPCRs as well as the identification of selective inhibitors of GPCR–G protein coupling. This point is critical should the GPCR mediate multiple functional responses via distinct G proteins.

Novel technologies have led to the development of GPCR–Gα fusion proteins in which the Gα component, normally interacting with the receptor, binds [^{35}S]GTPγS following agonist stimulation.[66,153,154] These fusion proteins provide several advantages for GPCR drug discovery. First, they eliminate the problem of whether the target cells expressing the recombinant GPCR under study have the appropriate endogenous G proteins that couple with the GPCR. This is a problem in the GPCR drug discovery field since different cell lines express different arrays of G proteins. As a consequence individual cell lines may not express the G proteins that naturally associate with a given GPCR. Furthermore, since recombinant GPCRs are usually overexpressed in cell lines used for drug discovery, the stoichiometric relationship of the GPCRs with the endogenous G proteins may be unnatural. This leads to diminished GPCR responses, as the appropriate G proteins with which the receptor normally interacts may not predominant and thus be unavailable to the receptor. Alternatively, the GPCR may be directed to interact with G proteins that predominant in the cell with which they do not normally interact. This could result in identification of compounds against recombinant receptors that may not interact with the endogenous receptors. By contrast, in the case of the GPCR–Gα fusion proteins, the GPCR cannot interact with the endogenous G proteins, and can only associate with the G protein to which it is fused. This results in a stoichiometry of GPCR to G proteins of 1:1, providing a more appropriate ratio of receptor to G protein to what one might expect to occur naturally.

Screening techniques have also been developed that employ such fusion proteins to measure dimer formation.[66,153,154] This was accomplished by fusing GPCRs to Gα mutants, which by themselves are unable to mediate GTP exchange. Alternatively, techniques have also been developed using mutant GPCRs that do not mediate agonist-induced GTP exchange in Gα and fusing that GPCR with a wild-type Gα. These functionally inactive mutant GPCR–Gα fusion proteins, expressed alone in cells, are unresponsive to agonist-induced [^{35}S]GTPγS binding. However, co-expression of the different mutant fusion proteins leads to reconstitution of a functional receptor dimer such that agonist binding to the GPCR dimer induces [^{35}S]GTPγS binding. Thus, [^{35}S]GTPγS binding only occurs when two different GPCR–G protein fusion proteins associate as a dimer. It is possible to employ this approach to measure homodimer formation, using dif-

ferent mutant forms of the same GPCR–G protein complex and probably can be used to measure heterodimer formation. As a consequence, this assay can be used to identify functional GPCR dimer formation and more importantly can be used to identify compounds that selectively interact with homo- versus heterodimers.

There are, nonetheless, disadvantages to employing the GTPγS binding assay to monitor GPCR activation. First, the assay primarily uses radioactive [^{35}S]GTPγS and as a consequence is expensive in the quantities used in HTS. Second, there are issues of high background signal in this assay, since [^{35}S]GTPγS will bind to all Gα in the tissues under study, in particular the small G proteins including Rho. For research purposes, this is mitigated somewhat by the use of antibodies that target the G protein in question and immunoprecipitate the Gα selectively binding [^{35}S]GTPγS. Based on this approach scintillation proximity assay formats have been set up to do this for drug screening.[155] However, since antibodies are employed, the technology is expensive when screening large libraries of compounds. Furthermore, different GPCRs have different levels of efficacy in terms of G protein coupling. As a result, one may observe varying levels of agonist-induced stimulation of [^{35}S]GTPγS binding that are highly dependent upon the receptor in question. Some of these problems can be overcome using GPCR–G protein fusion proteins. However, it is unclear if this approach is applicable to all GPCRs, since only a few GPCRs have studied by this technique.

Cell-based assays measuring second messengers and reporter gene expression

Although assays measuring binding of ligands to GPCRs have been used historically to identify GPCR drugs, most current technologies used for GPCR drug discovery are predominantly cell-based.[156] Cellular technologies examine receptors in an environment physiologically closer to that predicted for tissues. Thus, intact cells, rather than membranes, can provide functional readouts that are simpler to automate and quantitate. Indeed, most cell-based assays have now been developed to such a degree that they have been widely adopted into HTS programs of almost all drug discovery organizations.

The primary readout of such assays is accumulation of second messenger levels in response to GPCR activation (Fig. 5). The second messengers most commonly measured are cAMP, IP3, and Ca^{2+}.[157–160] Detecting these molecules measures functional responses of GPCRs coupled to the most predominant G proteins including Gα_s, Gα_i, Gα_o, and Gα_q. Using these assays, one can identify full agonists, partial agonists, and inverse agonists at both native and recombinant receptors.

The cellular levels of cAMP are dependent on the activity of adenylyl cyclase, which can be stimulated (via Gα_s) or inhibited (via Gα_i) by GPCRs. One can directly measure agonist-induced stimulation of adenylyl cyclase activity via Gα_s in broken cell preparations, but this assay format cannot easily detect inhibition of adenylyl cyclase occurring via Gα_i. Instead, in intact cells steady-state levels of cAMP, the end product of cyclase activity, are usually measured. The changes in levels of cAMP are induced in cells, and the second messenger is measured in lysates. Many commercial assays are now available to measure cAMP in lysates including radioimmunoassay approaches, time-resolved-FRET- and BRET-based assays, and those employing enzyme fragment complementation technology (Fig. 5).

The cAMP assays are limited to use for those GPCRs that couple to Gα_s or Gα_i. While stimulation of cAMP formation can be substantial, inhibition of cAMP levels by Gα_i-coupled receptors is variable, being dependent upon the basal level of cAMP formed. Thus, in many cases, inhibition is observed when adenylyl cyclase is activated either via stimulatory GPCRs or via agents that directly interact with the catalytic subunit of cyclase, such as forskolin. Inhibition can also be dependent on the appropriate Gα_i subtype being expressed in the cell line since GPCRs express preference for coupling to some but not necessarily all of the Gα_i subtypes (there are three) in a cell.

Activation of GPCRs coupled to G_q and G_o is measured by detecting cellular levels of IP3, the end product of activation of phosphoinositide phospholipase C.[39] While several radiometric assays are available to measure IP3, nonisotopic assays are primarily used for drug screening using an oxygen channeling assay (ALPHAscreen®, PerkinElmer) or fluorescent polarization technology. These approaches are competition assays in which free IP3 from the cell lysate competes with biotinylated IP3 for binding to an IP3 binding protein. Although the advantages of these technologies have been recently described elsewhere,[3] the transient nature of IP3 kinetics can make automation of these assays problematic. Consequently, a homogeneous assay has been developed for HTS using time-resolved-FRET techniques for the detection of other, more long-lived, inositol phosphates, such as inositol monophosphate.

Many GPCRs physiologically regulate changes in intracellular Ca^{2+} either through an IP3-mediated mechanism involving changes in stored Ca^{2+} or through the regulation of voltage-sensitive ionic conductance channels, which modulate Ca^{2+} influx. GPCRs can couple to a host of different G proteins that mediate these effects. However, effects on ionic conductance channels by GPCR activation are restricted to cells that express these channels. This is important because most cell lines used for drug discovery against GPCRs, such as human em-

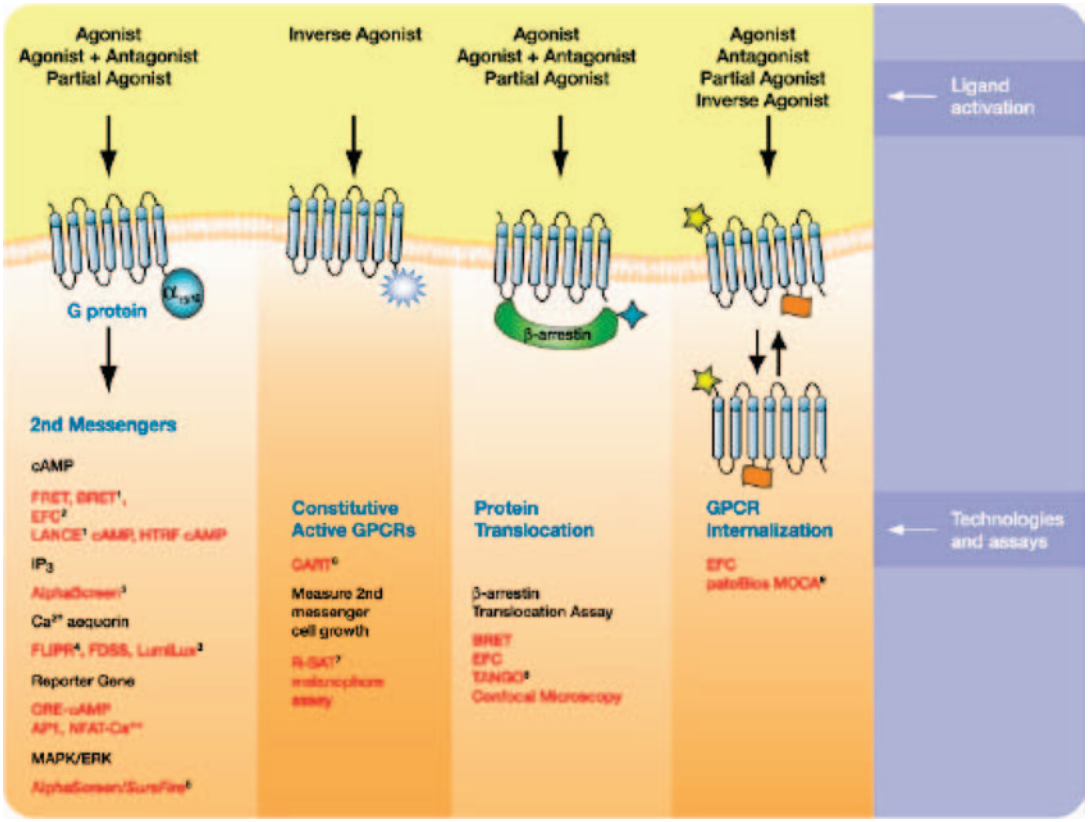

FIG. 5. Cell-based assays for GPCR drug discovery. A large number of different assay systems have been developed to facilitate the discovery of compounds targeting GPCRs. The vast majority of these technologies are cell based using either cell lines or primary cell types transfected to express recombinant GPCRs. Most technologies commercialized for GPCR drug discovery measure changes in second messenger levels as a response to GPCR activation (**first panel**). AP1 activator protein 1; CRE, cAMP response element; EFC, enzyme fragment complementation; FDSS, functional drug screening system; HTRF, homogeneous time-resolved fluorescence; NFAT, nuclear factor of activated T cells. These assays measure changes in cAMP, IP3, and Ca^{2+} using luminescent readouts. There are also assays to measure the MAPK, which is a convergent pathway for downstream GPCR signaling. Some assays that can be used to identify inverse agonists are those in which the GPCRs are forced to be constitutively active and in some cases use as a readout changes in cell growth or color as in the melanophore assays (**second panel**). CART, Constitutively Activated Receptor Technology; R-SAT, Receptor Selection and Amplification Technology. Assays purported to be able to measure activation of any GPCR are those that measure agonist-induced translocation of β-arrestin and can detect the translocation event using either confocal microscopy, BRET, or FRET technologies that measure protein–protein interaction (interaction of the GPCR and β-arrestin) or EFC technology (**third panel**). Finally, a technology proposed to detect the binding of any ligand to any GPCR is one in which the ligand-bound receptor is sequestered in the cell membrane and prevented from internalizing (**fourth panel**). This assay tags GPCRs with nuclear localization signals that normally target the receptor to the nucleus, and only when the receptor binds ligand (either agonist or antagonist) does the receptor remain at the cell surface. [1,3]Trademark of PerkinElmer (Waltham, MA). [2]Trademark of DiscoveRx Corp. (Fremont, CA). [4]Trademark of Molecular Devices Corp. (Sunnyvale, CA). [5]Trademark of TGR BioSciences (Thebarton [Adelaide], Australia). [6]Arena Pharmaceuticals (San Diego, CA). [7]Trademark of Acadia Pharmaceuticals (San Diego). [8]Trademark of Cell and Molecular Technologies (CMT), now part of Invitrogen Discovery Sciences (Madison, WI). [9]Multipurpose Original Cellular Assay (patoBios, Toronto, ON, Canada).

bryonic kidney 293 and Chinese hamster cells, do not express voltage-dependent Ca^{2+} channels at high levels. Ca^{2+} levels in cells are primarily measured using Ca^{2+}-sensitive dye, which changes its luminescent signal when it binds Ca^{2+}. Two of the most commonly employed instruments used to measure changes in Ca^{2+} are the fluorometric imaging plate reader (FLIPR™, Molecular Devices Corp., Sunnyvale, CA) or the functional drug screening system (FDSS, Hamamatsu Corp., Hamamatsu City, Japan), which use Fluo-3, Fluo-4, and Calcium 3 dyes.[161–165] In parallel, there has been rapid adoption of cell-based assays employing photoproteins, such as aequorin or photina, that enzymatically generate a luminescent signal upon elevations of intracellular calcium.[164,165] These latter techniques have the advantage that large signal-to-background ratios are generated in the assay, making them particularly useful for the detection of allosteric regulators, over and above the low false-pos-

itive rates from compound libraries observed using luminescent detection in comparison to fluorescent detection techniques.

These technologies can be used for drug discovery against orphan GPCRs as well as known GPCRs. For those receptors that are inefficiently coupled to Ca^{2+} transients, the assays can be modified to include either $G\alpha_{15/16}$ or various mutant forms of G proteins that couple effectively to phosphoinositide phospholipase C and have been shown to be promiscuous with regard to the GPCRs with which they interact.[166–169] Such G proteins induce artificial coupling of the target GPCR to the Ca^{2+} pathway and provide a more effective response to activation. However, it remains to be seen how effective this approach is for drug discovery against orphan GPCRs since directing GPCRs to associate with G proteins with which they do not normally interact can result in several artifacts—notably in the discovery of GPCR drugs that only act on the receptors in a screening assay and not in native tissues.

In addition to direct measurements of second messengers, several reporter gene assays are now available that detect the consequence of changes in second messengers. These HTS assays employ constructs transfected into cells consisting of second messenger response elements such as the cAMP response element or the calcium-sensitive activator protein 1 or nuclear factor of activated T cells elements linked to genes that encode for enzymes, such as luciferase or β-lactamase, that act to catalyze the formation of luminescent or fluorescent products.[157–159] This technology is highly sensitive to changes in GPCR-mediated changes in second messengers and is easily adapted to HTS format. However, the throughput assay system can be slow, since it requires the expression of protein. This can present problems since during the time needed to stimulate gene expression the GPCR may have desensitized. Therefore changes in gene expression may result from cells compensating for this effect rather than a response to the actual receptor stimulation. Furthermore, many drugs screened in this manner can produce off-target effects on the gene reporters *per se*. In addition, long-term compound exposure is sometimes toxic to the cells, resulting in false-positives during antagonist drug screening. Consequently, this approach requires many experimental controls to be employed to determine specificity of any "hit" identified.

More generally, a common problem with each of the assays to measure second messengers as a readout of GPCRs described above is that the compound tested may affect the second messengers independent of effects at the GPCR or indeed interfere with the response of the assay. This is a problem with many small molecule libraries in which the compounds exhibit internal fluorescence or quench the assay signal. As several of these issues can be clarified with either extensive controls or efforts to eliminate interfering compounds to reduce artifacts, such procedures are often expensive and laborious and may be cost prohibitive for small drug discovery companies.

Nonetheless, assays to measure second messenger accumulation are widely used for GPCR drug discovery. However, the applicability of these assays depends on the nature of the association of G protein with the GPCR. Thus, measurement of cAMP levels is most appropriate for GPCRs coupled to $G\alpha_s$ and $G\alpha_i$, while assays that measure either IP3 or Ca^{2+} are optimal for GPCRs coupled to $G\alpha_o$ and $G\alpha_q$. Thus, the use of the different HTS assays depends on *a priori* knowledge of the receptor G protein coupling and therefore can only be employed for limited sets of receptors.

Studies on the regulation of the MAPK system have indicated that most, if not all, GPCRs regulate this pathway. Here, GPCRs, including those linked to $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$, that modulate the activity of cAMP-dependent protein kinase and protein kinase C can influence the activity of Raf and Mek and subsequently ERK. In addition, any GPCR regulated by β-arrestin will also affect the activity of ERKs.[40] Therefore, the MAPKs are a point of convergence of the vast majority of GPCRs in regulating cell function, and measurement of ERK activity could serve as a universal assay for GPCRs. An ERK HTS assay has been developed for GPCRs that has been adapted and employed in a HTS format. The assay, termed AlphaScreen SureFire™ (TGR BioSciences, Thebarton [Adelaide], Australia) ERK, is a cell-based assay that combines a homogeneous, nonradioactive measurement of phosphorylated ERK1 and ERK2 using AlphaScreen technology. The approach can be used in cell lines as well as primary cells. An important advantage of the technology over other newer approaches that are proposed as universal GPCR assays (described below) is that it can be employed on endogenously expressed receptors as well as recombinant GPCRs.

Novel cell-based GPCR assays

Although measuring second messenger accumulation is the predominant approach used for GPCR drug screening today, other assay technologies have been now developed that provide some advantages over these classical assays (Fig. 5). For example, Acadia Pharmaceuticals (San Diego, CA) has developed a technology designated as Receptor Selection and Amplification Technology, an approach that measures receptor activation as a response to ability of GPCR ligands to modulate cell growth.[170–172] This technology, while proprietary and therefore used primarily at Acadia, has yielded interesting drugs, including inverse agonists at the serotonin 5-HT_{2A} receptor that are currently under development for central nervous system disorders.[170]

Furthermore, Arena Pharmaceuticals (San Diego) has developed a second technology, designated as Constitutively Activated Receptor Technology, used to identify ligands interacting with any GPCR.[173] Fundamentally, this technology involves inserting sequences into GPCRs known to induce constitutive activity. The goal of this approach is to generate constitutive activity into any target GPCR using these consensus sequences in order to identify inverse agonists. This approach has the potential to be used to screen orphan GPCRs. Arena also uses a melanophore assay for GPCR HTS[174] that measures changes in cell color as a response to GPCR activation.

Another different technology developed as a universal GPCR assay is one originally optimized by Lefkowitz, Caron, and colleagues at Duke University (Durham, NC) and then employed by Norak Biosciences (Research Triangle Park, NC) for drug discovery (now commercialized by Molecular Devices Corp.). This approach is based on the use of β-arrestin translocation in response to agonist stimulation. In fact, many different GPCRs respond to agonist stimulation with the β-arrestin translocation,[175,176] suggesting that this technology provides a universal assay to identify agonists at any GPCR. The technology was originally designed to measure β-arrestin translocation using confocal microscopy in which β-arrestin was tagged with green fluorescent protein and movement was subsequently detected with a fluorescent readout. The assay has been modified to be detected by BRET.[177–179]

More recently, Wehrman *et al*.[180] have developed an enzyme fragment complementation assay to measure β-arrestin translocation in an assay format adapted for microtiter plate detection instruments used in HTS. Here, two fragments of the enzyme β-galactosidase are employed: a small N-terminal fragment (Prolink) and a large truncated form of β-galactosidase (enzyme acceptor). Each fragment alone is inactive, but when added together they recombine to form an active β-galactosidase enzyme that can produce thousand of luminescent molecules in a short time. In this assay, Prolink is tagged to the GPCR C-terminus, and the enzyme acceptor is tagged to β-arrestin. When agonist stimulates the GPCR, the β-arrestin–enzyme acceptor associates with the receptor–Prolink, and complementation occurs, generating a highly luminescent response. An advantage of this technology is the extreme sensitivity since, instead of a single luminescent response as seen with FRET or BRET or confocal microscopy, this response involves the formation of thousands of luminescent molecules.

O'Dowd *et al.*[181] at the University of Toronto (Toronto, ON, Canada) and the biotechnology company patoBios Ltd. (Toronto) have developed a GPCR internalization assay useful to identify any ligand, including antagonists, that binds to a GPCR. They and others have shown that GPCRs can internalize not only to clathrin-coated vesicles but also to the nucleus.[182–187] Such internalization of GPCRs is facilitated by insertion of a nuclear localization signal into the cytoplasmic domains of the receptor. Thus, in the absence of ligand, these GPCRs remain in the cytosol or nucleus. This group[181] found that binding of agonists and antagonists induces GPCRs retention at the cell membrane. Consequently, ligand binding is measured by the amount of GPCR expression at the cell surface. This assay was adapted using β-galactosidase enzyme fragment complementation technology to measure surface expression of the receptors and has been shown to work for several GPCRs, being selective, easy to use, and adaptable to HTS assay formats. Since the assay does not require the receptor to be coupled to G protein, it can be used to identify antagonists.

Most other cell-based assays used to screen for GPCR drugs require agonist activation of the receptor with antagonists being identified by their ability to inhibit the agonist activation. However, in the absence of agonist, most assays do not allow one to identify an antagonist. The patoBios technology[181] may be the first cell-based assay allowing for identification of GPCR antagonists in the absence of known agonists. This attribute is particularly important for drug discovery against orphan GPCRs because identification of antagonists is essential in determining the functioning of the receptors and for establishing agonist specificity. Furthermore, O'Dowd *et al.*[181] have also found that GPCR heterodimers also internalize. Thus, if one tags one GPCR monomer with a nuclear localization signal and the other monomer does not have a nuclear localization signal, both receptors can internalize. Ligands binding to the nontagged receptor thus act to retain the dimer at the cell membrane, and surface expression is detected using standard enzyme fragment complementation assays. Consequently, this technology may be used to identify ligands that bind to the heteromer and distinguish ligands selective for either the homo- or heterodimer complexes.

Conclusions

The central role of GPCRs in human physiology and the extensive diversity in function have made the GPCR family one of the prime targets for drug discovery. Many GPCRs have discrete biological roles, indicating that drugs targeting those proteins will produce specific therapeutic effects. Importantly, it is known that GPCR-directed drugs provide good therapeutics and can treat numerous diseases and disorders for which there are few, if any, alternatives.

Advances made in the GPCR research field in the last decade have heightened interest in discovering new GPCR drugs. For example, the finding that some recently de-orphanized GPCRs have powerful biological func-

tions has led to major attempts to better understand the roles of the hundreds of remaining orphan GPCRs, thereby identifying those receptors with the potential to be targets for novel therapeutics. These receptors thus represent a relatively unexplored field of drug discovery yet may have high value for the pharmaceutical industry, partly because of the large number of targets available as well as the unique functions they probably mediate.

The identification of drugs targeting orphan GPCRs, inverse agonists, allosteric regulators, pharmacological chaperones, and hetero-oligomers, while providing important new directions for drug discovery, has also directed the industry to develop novel technologies for HTS. One may not easily employ classical drug discovery approaches to identify drugs targeting orphan GPCRs or to identify pharmacological chaperones; certainly they cannot be easily used to identify hetero-oligomer-selective drugs. Historically, the industry has streamlined cell-based assay approaches to measure second messenger accumulation or MAPK activity to discover drugs against most if not all known GPCRs. However, novel technologies measuring protein–protein interactions have only been more recently developed. Collectively, these approaches can be used for discovery of almost any type of compound against almost any GPCR in the human genome.

Many of these approaches have now been incorporated into HTS formats and are consequently becoming more commonly employed for drug discovery. Importantly, novel technologies that measure subtle conformational changes in GPCRs are also emerging using physical techniques,[188,189] including dielectric spectroscopy, optical surface plasmon resonance biosensing, isothermal titration calorimetry, second harmonic generation, and differential scanning calorimetry. All of these techniques can be employed to identify allosteric regulators, as well as agonists acting at the same receptor yet producing different functional responses.

With such technologies, when optimized for HTS purposes, one anticipates that they will provide a foundation for the development of a new generation of drugs, potentially with therapeutic properties beyond those GPCR drugs presently available.

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