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THE ORIGINS OF DIVERSITY AND SPECIFICITY IN G PROTEIN-COUPLED RECEPTOR SIGNALING

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ABBREVIATIONS:
EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinases 1 and 2; GPCR, G protein-coupled receptor; G protein, heterotrimeric guanine nucleotide-binding protein; GEF, guanine nucleotide exchange factor; MAP, mitogen-activated protein; NHERF, Na^+/H^+ exchanger regulatory factor; PDZ, Post synaptic density of 95 kDa-disc large-zona occludens; RAMP, Receptor Activity Modifying Protein; RCP, Receptor Component Protein

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ABSTRACT

The modulation of transmembrane signaling by G protein-coupled receptors (GPCRs) constitutes the single most important therapeutic target in medicine. Drugs acting on GPCRs have traditionally been classified as agonists, partial agonists, or antagonists based on a two state model of receptor function embodied in the ternary complex model. Over the past decade, however, many lines of investigation have shown that GPCR signaling exhibits greater diversity and 'texture' than previously appreciated. Signal diversity arises from numerous factors, among them the ability of receptors to adopt multiple 'active' states with different effector coupling profiles, the formation of receptor dimers that exhibit unique pharmacology, signaling, and trafficking, the dissociation of receptor 'activation' from desensitization and internalization, and the discovery that non-G protein effectors mediate some aspects of GPCR signaling. At the same time, clustering of GPCRs with their downstream effectors in membrane microdomains, and interactions between receptors and a plethora of multidomain scaffolding proteins and accessory/chaperone molecules confers signal preorganization, efficiency, and specificity. In this context, the concept of agonist selective trafficking of receptor signaling, which recognizes that a bound ligand may select between a menu of 'active' receptor conformations and induce only a subset of the possible response profile, presents the opportunity to develop drugs that change the quality as well as the quantity of efficacy. As a more comprehensive understanding of the complexity of GPCR signaling is developed, the rational design of ligands possessing increased specific efficacy and attenuated side effects may become the standard mode of drug development.

INTRODUCTION

The heptahelical G protein-coupled receptors (GPCRs) constitute the most diverse form of transmembrane signaling protein. An estimated 1% of the mammalian genome encodes GPCRs, and about 450 of the approximately 950 predicted human GPCRs are expected to be receptors for endogenous ligands (Takeda et al., 2002). GPCRs detect an extraordinarily diverse set of stimuli in the external environment, from photons of light and ions to small molecule neurotransmitters, peptides, glycoproteins, and phospholipids. What's more, nearly 40% of all current therapeutics target GPCRs (Brink et al., 2004).

The mechanism by which GPCRs transduce extracellular messages into intracellular cellular responses was initially envisioned as a simple linear model in which agonist binding promotes transition of the receptor from an 'off' to an 'on' state capable of engaging heterotrimeric guanine nucleotide-binding (G) proteins, whose dissociated Gα and Gβγ subunits in turn activate or inhibit various downstream effector molecules. Implicit in this model is the concept that a GPCR agonist will impact every consequence of receptor activation in the same fashion, whether G protein coupling, receptor desensitization, internalization or trafficking. Recently, however, experimental evidence documenting the existence of multiple 'active' receptor states, alternative mechanisms of signaling, and preogranization of GPCR signaling units has dramatically expanded our notions of the complexity and texture of GPCR signaling and forced a reexamination of even the fundamental concepts of agonism and antagonism. It is increasingly clear that for a given GPCR, the optimal receptor conformation for G protein activation differs between G protein pools, and that synthetic, and in some cases naturally-occurring, ligands can selectively promote different coupling conformations of the receptor (vide infra). Many examples now exist of 'agonists' that activate only a subset of potential G protein partners or induce G protein coupling without triggering desensitization and endocytosis, or of 'antagonists' that cause receptor desensitization or that initiate apparently G protein-independent signals without producing detectable activation of heterotrimeric G proteins. Here, we examine current insights into the source of GPCR signaling diversity and specificity, and discuss the impact of these factors on the classical concepts of agonism and the process of drug discovery.

THE EVOLUTION OF RECEPTOR THEORY

The ternary complex model of GPCR function. GPCRs transmit signals intracellularly by functioning as ligand-activated guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins. G protein activation is initiated through hormone-driven changes in the tertiary structure of the transmembrane heptahelical receptor core that are transmitted to the intracellular transmembrane loops and carboxyl terminus. These conformational changes alter the ability of the receptor to interact with intracellular G proteins and catalyze the exchange of GDP for GTP on the heterotrimeric G protein alpha subunit. The GTP-bound alpha subunit stimulates its cognate downstream effectors, *e.g.* an adenylate cyclase or phospholipase C, conveying information about the presence of an extracellular stimulus to the intracellular environment.

In the simplest conceptualization, a membrane receptor functions as a switch, existing in either an empty "off" state or an agonist-bound "on" state. Such early allosteric models of membrane receptor function were introduced in the late 1960s (Karlin, 1967). Evidence that GPCR behavior was more complex originated with the finding that β-adrenergic receptors exhibit two affinity states for agonists, the relative proportions of which are modulated by the presence of guanine nucleotides (DeLean et al., 1980). The model advanced to explain these phenomena predicted that in the presence of GDP, agonist binding promotes the formation of a long-lived ternary complex between agonist (H), GPCR (R), and heterotrimeric G protein (G) that exhibits high agonist binding affinity. In the absence of the G protein, or when the presence of GTP allows for receptor-catalyzed G protein activation, the H-R-G complex is dissociated and the receptor resides in a low affinity (H-R) state.

The extended ternary complex model. Efforts to employ receptor chimeras to explore the structural basis of specificity in receptor-G protein coupling led to the unexpected finding that α1b adrenergic receptors carrying relatively conservative substitutions of β2 adrenergic receptor-derived amino acid sequences in the C-terminal portion of the third intracellular loop activate Gq/11 proteins in the absence of agonist. Indeed, mutation of a single residue, Ala293, of the α1b-adrenergic receptor to any other amino acid increased the agonist affinity of the receptor and produced constitutive stimulation of

phosphatidylinositol hydrolysis (Kjelsberg et al., 1992). Using analogous constitutively active mutants of the β2-adrenergic receptor, it was subsequently possible to demonstrate that some ligands that appear to be classical competitive antagonists on native receptors show selective high affinity for the inactive receptor and suppress basal receptor activity (Samama et al, 1994). Ligands possessing these properties have been termed "negative antagonists" or "inverse agonists".

This, and subsequent work involving a large number of GPCRs, has affirmed the hypothesis that the receptor exists in spontaneous equilibrium between two conformations (active: R*; inactive: R) that differ in their ability to activate G proteins (Samama et al., 1993). In the native state the receptor is maintained predominantly in the R conformation by intramolecular interactions within the transmembrane helical bundle, i.e. the spontaneous equilibrium heavily favors the inactive R state. Agonist binding, or selective mutagenesis, relieves these constraints, allowing the receptor to 'relax' into the R* conformation that enables G-protein coupling. The extended ternary complex model developed to explain these phenomena proposes that the intrinsic efficacy of a ligand is a reflection of its ability to alter the equilibrium between R and R* (Lefkowitz et al., 1993). According to the model, *Full agonists* stabilize the R* conformation, pulling the equilibrium toward the active state to generate full receptor activation and a maximal response; *Partial agonists* have lower intrinsic efficacy than full agonists, thus producing a submaximal system response and potential attenuation of full agonist activation; *Antagonists* bind indiscriminately to both R and R* producing no physiological response but blocking the response to agonists; *Inverse agonists* act as antagonists in non constitutively-active systems, but have the added property of actively reducing receptor-mediated constitutive activity of GPCR systems by binding preferentially to R and pulling the equilibrium toward the inactive state. Even the behavior of "Protean agonists", ligands that act as partial agonists in some systems and as inverse agonists in others, can be accounted for within the extended ternary complex model if one assumes that the active receptor conformation produced by ligand binding (H-R*) is of a lower efficacy that the spontaneously formed R* state (Kenakin, 1995a). Under conditions of low basal activity, i.e. little or no spontaneously formed R*, such a ligand would behave

as a partial agonist, while under conditions of high basal activity it would behave as an inverse agonist.

Three state to multi-state models. While the ternary complex model can sufficiently explain the properties of agonism, antagonism, partial agonism, and inverse agonism, it is still limited in that it accommodates the existence of only two functional receptor states. However several lines of experimental evidence suggest that multiple active states of GPCRs can exist.

Many GPCRs, either at physiologic levels or when overexpressed, are promiscuous, i.e. they stimulate different signaling pathways by activating more than one G protein pool. In a two state model, where only a single R* conformation exists, the agonist pharmacology of a receptor should be the same regardless of the response being measured. Yet a paradoxical reversal of relative efficacy of agonists has been described for several GPCRs that activate more than one stimulus-response element, including the 5-HT2c receptor (Berg et al., 1998), pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (Spengler et al., 1993), dopamine D2 receptor (Meller et al., 1992), and neurokinin NK-1 receptor (Sagan et al., 1999). Although differential stimulus pathway activation can occur through a strength of signal type of mechanism, i.e. a highly efficacious agonist might activate two pathways whereas a weaker agonist may activate only the more sensitive one, the reversal of the relative efficacy of different agonists acting on the same receptor cannot be explained on the basis of a two state model.

The demonstration that GPCRs exhibit ligand-specific activation states led to the proposal that three or even more active states of the same receptor may exist. In these three-state or multistate models, certain agonists are predicted to induce distinct “active” conformations of the receptor by differentially exposing regions of the intracellular domains involved in coupling to different G protein pools. Indeed, multiple G protein-coupled states of the α_2-adrenergic receptor can be distinguished using a variety of guanine nucleotide analogues (Seifert et al., 1999). Similarly, several receptor mutations have been described that produce constitutive activity that is restricted to a single signaling pathway among those ordinarily activated by the receptor (Perez et al., 1996).

These mutations presumably restrict conformational isomerization of the receptor to a certain subset that promotes specific G protein coupling conformations. While the behavior of a mutated receptor cannot be extrapolated a priori to its wild type counterpart, these data clearly demonstrate that subtle changes in receptor structure outside of the G protein-coupling domains, as might occur upon binding different agonist ligands, can alter G protein selectivity (Kenakin, 2002).

Biophysical evidence also supports the concept that different GPCR ligands induce distinct populations of receptor microconformation (Ghanouni et al., 2001). Fluorescence lifetime spectroscopy of β2 adrenergic receptors fluorescently labeled at Cys265 reveals a Gaussian distribution of environments for the probe reflecting continuous fluctuations in receptor conformation. Addition of agonist or antagonist ligands changes the distribution of receptor conformations, reflecting the stabilization of a specific subset of conformations. Moreover, different agonists select different arrays of receptor conformation, consistent with the induction of ligand-selective active states.

The existence of multiple active receptor conformations makes it plausible that agonists can change not only the degree, but also the 'quality' of receptor activation. It is known that different areas of the cytosolic loops on receptors activate different G-proteins (Wade et al., 1999). It is thus predictable that agonists producing distinct tertiary conformations of a receptor could expose these different G-protein-activating sequences so as to produce differential activation of G proteins. This multi-state model of GPCR activation provides the theoretical basis for the concept of signaling-selective agonism, also referred to as 'agonist-specific trafficking of receptor signaling' (Kenakin, 1995b; Kenakin, 1995c).

THE ORIGINS OF GPCR SIGNALING DIVERSITY

In contrast to the large number of GPCR sequences in the genome, there are comparatively few genes encoding heterotrimeric Gα subunits. With input from a large number of receptors converging on a limited number of transducer elements, how do GPCRs generate diverse responses under different conditions and in different tissues?

Diversity in a two-state model. Numerous factors expand the signaling repertoire of heptahelical GPCRs. First is the sheer complexity of the receptors themselves. The majority of GPCR families consist of multiple receptor subtypes, often with different G protein coupling specificities. For example, there are at least 12 different mammalian genes encoding serotonin receptors. Additional complexity derives from alternative splicing of receptor genes and RNA editing, generating multiple receptor isoforms with distinct biochemical properties from the same gene (Paasche et al., 2001).

Another layer of complexity arises from the ability of each G protein class to activate multiple downstream effectors. Both Gα and Gβγ subunits contribute to the modulation, in a synergistic or antagonistic fashion, of either the same or unrelated effectors, resulting in dual intracellular signaling. An example is the simultaneous Gi/o-mediated inhibition of adenylate cyclase via the Gα subunit and stimulation of Phospholipase Cβ via the Gβγ subunit (Exton, 1996). Further complexity arises from secondary modulation of intracellular effectors, for example the indirect activation of phospholipase A2 following a rise in intracellular Ca^{2+} concentration (Clark et al., 1991).

Finally, there is the capacity for simultaneous activation of multiple G protein pools. Some Gi/o-coupled receptors, for example, mediate phosphoinositide hydrolysis through a pertussis toxin-insensitive pathway in addition to mediating pertussis toxin-sensitive inhibition of adenylate cyclase (Jones et al., 1991). Quantitative analysis of agonist-induced guanine nucleotide exchange on Gα-subunits has provided more definitive evidence. The dual coupling to Gs and Gq/11 family G proteins (Jin et al., 2001) or to Gi/o and Gq/11 family G proteins (Offermanns et al., 1994) has now been reported for many GPCRs. In some cases, a single receptor has been found to simultaneously activate members of three or even four unrelated classes of G protein (Gs, Gi/o, Gq/11, and G12) (Laugwitz et al., 1996).

A persistent question is whether such multiple G protein coupling represents pleiotropy, i.e. physiologic activation of different G protein species, or promiscuity, i.e. low efficacy activation of non-preferred G protein species as a result of receptor or G protein overexpression. In experimental systems, an agonist activating one GPCR that stimulates multiple G proteins frequently elicits signals downstream of each G protein with differing efficacy and/or potency (Offermanns et al., 1994). Unless there is reversal

of agonist efficacy, such behavior is consistent with a two state model in which the receptor can interact with both preferred and secondary transducers. Indeed, emergence of a dual signaling commonly occurs as the level of receptor expression increases suggesting that most GPCRs are promiscuous (Cordeaux et al., 2000; Zhu et al., 1994). Similar phenomena arise from changes in the expression levels of the participating G proteins (Nasman et al., 2001). On the other hand, many studies have demonstrated dual or multiple coupling in systems where the GPCR is constitutively expressed at low levels, consistent with physiologically relevant pleiotropic G protein coupling (Jin et al., 2001; Laugwitz et al., 1996).

Diversity due to multiple receptor conformations. Consistent with models of agonist-specific trafficking of receptor signaling, a number of structurally modified agonists for promiscuous peptide and non-peptide GPCRs have been shown to promote selective G protein coupling (Chakrabarti et al., 1995; MacKinnon et al., 2001; Spengler et al., 1993). A similar phenomenon is signal-selective antagonism, in which an antagonist blocks only a subset of the signaling pathways elicited by an agonist. This has been clearly described for the cholecystokinin CCK-B (Pommier et al., 1999) and neurokinin NK-1 receptors (Sagan et al., 1996).

Other examples of ligand-selective GPCR regulation include ligands that promote coupling to one G protein pool while antagonizing coupling to another. The gonadotropin-releasing hormone (GnRH) receptor 'antagonist' Ant135-25 acts as an antagonist with respect to Gq-coupling by the GnRH receptor, but functions as an agonist in cellular contexts where the receptor is coupling to Gi (Maudsley et al., 2004). Similarly, the β2-adrenergic receptor 'antagonist' ICI-118-551 [(±)-1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride], which behaves as an inverse agonist for coupling to Gs, was recently found to act as an agonist for β2-adrenergic receptor coupling to Gi (Gong et al., 2002).

Studies of agonist-induced GPCR desensitization and endocytosis have likewise demonstrated the existence of ligand-specific receptor conformations. In a two-state model, it would be expected that the relative propensity of agonists to induce desensitization would parallel their relative efficacy for signaling. For μ opioid receptor

agonists this is generally true, with the notable exceptions of methadone, L-α-acetyl methadone, and buprenorphine, which induce disproportionate receptor phosphorylation and desensitization (Yu et al., 1997). Similarly, both enkephalins and morphine stimulate δ and μ opioid receptors, but only enkephalins induce rapid receptor internalization (Keith et al., 1996). Disparities between primary pathway activation and desensitization have been also demonstrated for neurokinin NK-1 (Maudsley et al., 1998) and serotonin 5-HT_{2C} receptors (Stout et al., 2002). Studies of the recovery from desensitization also suggest that agonists differentially affect receptor conformation. Whereas the resensitization of 5-HT_3 receptors after prolonged stimulation with partial agonists is mono-exponential, desensitization induced by full agonists recovers with sigmoid kinetics, suggesting at least 3 transitional steps and up to 4 states (van Hooft et al., 1996). Even more dramatic are GPCR 'antagonists' that stimulate receptor internalization. The cholecystokinin (CCK) receptor antagonist D-Tyr-Gly-[(Nle28,31,D-Trp30)cholecystokinin-26–32]-phenethylester, which blocks CCK-mediated G protein activation, nonetheless causes profound receptor internalization (Roettger et al., 1997). Certain competitive antagonists of the angiotensin AT1a receptor, such as [Sarcosine1, Ile4, Ile8] Ang II, exhibit similar properties (Holloway et al., 2002).

The finding that synthetic ligands can induce two or more functionally distinct receptor conformations suggests the possibility that native hormones interacting with the same GPCR may exhibit agonist-specific trafficking. Does this phenomenon occur in nature? Some data suggest so. For example, fully processed follicle-stimulating hormone, a glycopeptide, has different effects on adenylate cyclase and phospholipase C activity than the less abundant circulating nonglycosylated form of FSH, which can even act as a receptor antagonist. These data suggest that post-translational processing in the gonadotrope modulates signaling by an endogenous peptide agonist (Arey et al., 1997). A more compelling case can be made for the Chemokine Type 7 (CCR7) receptor. CCL19 and CCL21, two endogenous CCR7 ligands that exhibit equivalent potency and efficacy with respect to calcium mobilization differ dramatically in terms of their ability to cause receptor phosphorylation and desensitization. Moreover, only the 'desensitizing' ligand, CCL19, robustly activates the mitogen-activated protein (MAP) kinases,

extracellular signal-regulated kinases 1 and 2 (ERK1/2), hinting that desensitization itself may be a discretely regulating signaling event (Kohout et al., 2004).

Diversity arising from receptor dimerization. Coprecipitation studies, complementation experiments using mutated or chimeric receptors, and fluorescence energy transfer measurements all support the hypothesis that many, if not most, GPCRs can form homodimers, heterodimers, or higher order multimers (Angers et al., 2002). The assembly of receptor multimers establishes another level of conditioning that can affect GPCR ligand recognition, signaling, and intracellular trafficking. In the limit case, receptor dimerization is a prerequisite for the functionality of the receptor. The γ-amino butyric acid type B ($GABA_B$)R1 and $GABA_B$R2 receptors are nonfunctional as monomers. Only $GABA_B$R1-R2 heterodimers, which assemble in the endoplasmic reticulum-Golgi apparatus, are capable of membrane expression and signaling (Jones et al., 1998).

Numerous, less dramatic examples exist wherein dimerization alters receptor pharmacology and signaling. Dimerization of the μ and δ opioid receptors decreases the affinity for certain agonists, presumably by altering the conformation of extracellular receptor domains (George et al., 2000). The converse is true for heterodimers of the adenosine A2A and dopamine D1 receptors, where selective agonist affinities are increased (Franco et al., 2000). Agonist efficacy can also be altered by GPCR dimerization. For example, heterodimerization between somatostatin SSTR5 and SSTR1 (Rocheville et al., 2000) and also between μ and δ opioid receptors (George et al., 2000), increases both the intrinsic efficacy and the apparent potency of some agonists.

Cross talk between heterodimeric GPCR pairs can positively or negatively modulate the response to agonists binding to either member of the pair, resulting in either enhanced G protein activation or cross-inhibition (Ferre et al., 1998; Jordan and Devi, 1999). Even qualitative changes in G protein-coupling specificity have been reported. Whereas μ and δ opioid receptors couple to pertussis toxin-sensitive G-proteins when expressed individually, co-expression of these receptors results in opioid signaling in the presence of pertussis toxin, suggesting that μ-δ heterodimers can activate pertussis toxin-insensitive G proteins (George et al., 2000). Finally, heterodimerization can affect

receptor desensitization and trafficking, thus modulating the duration of GPCR signaling. For example, the nonselective opioid agonist etorphine, which causes internalization of δ, but not κ opioid receptors, does not cause δ opioid receptor internalization when it is coexpressed with the κ receptor (Jordan and Devi, 1999).

Non-receptor modifiers of GPCR signaling. The pharmacology of at least two GPCRs is determined not exclusively by the intrinsic structure of receptor, but by their interaction with the nonreceptor RAMP (Receptor Activity Modifying Protein) and RCP (Receptor Component Protein) proteins (McLatchie et al., 1998; Evans et al., 2000). RAMPs form complexes with the calcitonin receptor-like receptor (CRLR) and calcitonin receptor and control receptor trafficking and function. RAMP binding to the CRLR is required for transport of nascent receptors to the plasma membrane. Further, the specific CRLR-RAMP complex determines the ligand specificity of the receptor. The CRLR-RAMP1 complex acts as a receptor for the calcitonin gene-related peptides, a pleiotropic family of neuropeptides with homology to calcitonin, amylin and adrenomedullin. When CRLR is coexpressed with RAMP2 and RCP it functions as an adrenomedullin receptor. Similarly, complexes between a naturally occurring splice variant of the calcitonin receptor and RAMP1 or RAMP3 yields a functional amylin receptor. RAMP expression is modified under physiologic stress and in response to glucocorticoids, suggesting that cellular responsiveness to certain hormones can be regulated through the control of accessory protein expression.

Desensitization as a modifier of signal quality. Mechanisms to dampen GPCR signals exist at every level, from receptor to G protein and effector. At the receptor level, two processes, termed heterologous and homologous desensitization, respectively, have been shown to play a role not only in limiting signal duration and intensity, but also in the determination of signal quality. Both processes are initiated by receptor phosphorylation. The activation of second messenger-dependent protein kinases, such as protein kinase A and protein kinase C, leads to phosphorylation of serine and/or threonine residues in the cytosolic loops and C-terminal tail of many GPCRs. Phosphorylation of these sites is sufficient to inhibit receptor-G protein coupling, and agonist occupancy is not required.

Thus, unliganded receptors, even those for other ligands, can be desensitized in this manner. In contrast, homologous desensitization is specific for agonist-occupied GPCRs. It is a two-step process in which the receptor is first phosphorylated by one of a family of G protein-coupled receptor kinases (GRKs), then binds to an arrestin protein that exhibits high affinity only for the agonist-occupied, GRK-phosphorylated form of the receptor (Stoffel et al., 1997). Arrestin binding serves to both to sterically inhibit G protein coupling and to target the receptor to clathrin-coated pits for internalization (Ferguson, 2001).

The observation that Protein Kinase A phosphorylation of small peptides derived from the intracellular loops of the β2-adrenergic receptor converts them from *in vitro* activators of Gs to activators of Gi, suggested the hypothesis that heterologous desensitization might cause a 'switch' in β2-adrenergic receptor-G protein coupling selectivity. Using both Protein Kinase A phosphorylation-site mutants expressed in cellular systems and purified recombinant receptors and G proteins *in vitro*, it has been possible to demonstrate that Protein Kinase A phosphorylation of the β2-adrenergic receptor both impairs Gs coupling and promotes receptor coupling to Gi (Daaka et al., 1997; Zamah, et al., 2002). Not only does 'switching' enhance desensitization by converting the receptor from an activator to an inhibitor of adenylyl cyclase, it confers novel signaling properties on the receptor, such as the ability to stimulate Gi-dependent ERK1/2 activation. An analogous mechanism has been described for the Gs-coupled murine prostacyclin receptor. In this case, Protein Kinase A phosphorylation of S357 of the receptor is required for receptor coupling to Gi and Gq/11, but not to Gs (Lawler et al., 2001). Other GPCRs demonstrate type selective desensitization of G protein coupling following Protein Kinase A or Protein Kinase C activation. For example, Gq/11-mediated glutamate release by the subtype 1a metabotropic glutamate receptor (mGluR1a) is progressively desensitized by Protein Kinase C-mediated receptor phosphorylation, while a simultaneous inhibitory signal mediated through Gi/o coupling remains unaffected. The result is that in the presence of a persistent stimulus, the mGluR1a receptor switches from an activator to an inhibitor of glutamate release (Herrero et al., 1998). Collectively, these data suggest that regulation of the G protein coupling specificity by receptor phosphorylation adds an additional level of control that

permits the temporal resolution of cellular signaling elicited during the sustained stimulation of a receptor.

GPCR coupling to non-G protein effectors. A final source of GPCR signaling diversity arises from data suggesting that GPCRs transmit 'G protein-independent' signals, and that coupling to certain non-G protein effectors exhibits features consistent with agonist-specific trafficking.

The intracellular domains of several GPCRs have been shown to bind to proteins that might function as alternative GPCR signal transducers, among them GEFs for small G proteins, nonreceptor tyrosine kinases, and several proteins that function as adaptors or scaffolds. A specific peptide motif in the C-terminus of the β1-adrenergic receptor binds directly to the Post synaptic density protein of 95 kDa (PSD95)-disc large-zona occludens (PDZ) domain of the cAMP-regulated Ras GEF (CN-Ras GEF), allowing the receptor to stimulate guanine nucleotide exchange on the small G protein, Ras (Pak et al., 2002). Another PDZ-domain containing protein, the Na^+/H^+ exchanger regulatory factor/ezrin binding protein 50 (NHERF/EBP50) binds to the C-terminus of β2 adrenergic and parathyroid hormone PTH/PTHrP receptors and confers the ability to regulate NHE3 activity (Hall et al., 1998; Mahon et al., 2002). Stimulation of the JAK-STAT pathway of transcriptional regulation by angiotensin AT1a receptors involves tyrosine phosphorylation of AT1a receptor tail by a Src family kinase, followed by association of JAK2 with the receptor (Ali et al, 1997).

However the most compelling evidence to date for 'G protein-independent' signaling involves the utilization of arrestins as alternative signal transducers. The two non-visual arrestin isoforms (β-arrestin 1 and 2) can bind to several signaling proteins and recruit them to agonist-occupied GPCRs (Luttrell and Lefkowitz, 2002). Src family nonreceptor tyrosine kinases (Luttrell et al., 1999), components of the c-Jun N-terminal kinase 3 (JNK3) and ERK1/2 MAP kinase cascades (McDonald et al., 2000; DeFea et al., 2000), the E3 ubiquitin ligase, mdm2 (Shenoy et al., 2001), and the PDE4D3 and PDE4D5 isoforms of cAMP phosphodiesterase (Perry et al., 2002) are recruited to GPCRs in this manner. In this distinctive model of GPCR signaling, β-arrestin binding is thought to confer enzymatic activity upon the receptor at the same time that it uncouples

the receptor from its cognate G proteins. Indeed, the finding that arrestin-bound M2 muscarinic acetylcholine receptors exhibit increased affinity for agonists, but not antagonists, has led to speculation that the agonist-receptor-arrestin complex represents an 'alternative ternary complex' (Gurevich, et al., 1997).

Evidence that arrestin-bound GPCRs signal independently from G protein activation comes from several sources. Stimulation of a G protein-uncoupled DRY/AAY mutant of the AT1a receptor with angiotensin II fails to induce detectable G protein loading, but still promotes β-arrestin 2 recruitment, receptor internalization, and ERK1/2 activation that is abolished when β-arrestin 2 is selectively depleted by RNA interference (Wei et al, 2003). In an analogous fashion, exposure of the wild type AT1a receptor to the antagonist [Sarcosine1, Ile4, Ile8] AngII induces β-arrestin 2 recruitment and ERK1/2 activation in the absence of detectable G protein activation. This signal too is abolished by depletion of β-arrestin 2 by RNA interference. Analogous results have been reported for the β2-adrenergic receptor, where the inverse agonist, ICI118551, acts as a partial agonist for ERK1/2 activation (Azzi et al., 2003). The ERK1/2 response is absent in β-arrestin 1/2 null murine embryo fibroblasts, but can be restored by expression of β-arrestin 2. This β-arrestin-dependent ERK1/2 activation has a slower onset and more sustained duration that G protein-mediated ERK1/2 activation (Ahn et al., 2004). For GPCRs, like the NK-1, AT1a, and V2 vasopressin receptors, all of which form stable GPCR-arrestin complexes, the β-arrestin-dependent 'signalsome' appears to remain intact as the receptor transits the endosomal compartment, resulting in activation of a spatially-constrained, extranuclear pool of activated ERK1/2 (DeFea et al., 2000; Tohgo et al., 2002). In contrast, G protein-dependent ERK1/2 activation tends to promote nuclear translocation of the kinase and ERK1/2-dependent transcriptional responses. Thus signal strength, duration, subcellular localization, and functional consequence are all dictated by the mechanism of signal propagation.

THE ORIGINS OF SIGNALING SPECIFICITY

The converse of signaling diversity is signaling specificity, the constraint of responses to specific cells or tissues, even when the stimulus itself may be systemic, as in the case of a

circulating hormone. In essence, there must be mechanisms to limit 'signal spread' and work against promiscuity.

There are numerous factors, both intracellular and extracellular, within the classical conceptualization of GPCR signaling that promote specificity. For example, the highly localized release and rapid reuptake or extracellular degradation of neurotransmitters within the synaptic space provides a highly effective means of confining signals spatially. For systemic hormones, tissue selective expression of receptor subtypes comes into play. A typical cell may express more than ten different GPCR genes, different combinations of G protein subunits, and multiple isoforms of effector molecules. The differential expression of these various proteins imposes signal specificity at many levels, resulting in hormone responses that are customized for the specific cell type. Signal duration and intensity are selectively modulated through rapid receptor desensitization and internalization, and more slowly by downregulation of receptor expression (Ferguson, 2001). Nonetheless, GPCR signaling systems appear to exhibit higher levels of preorganization than can be accounted for by control of ligand availability and tissue-specific expression of transducer elements. Numerous studies have indicated that different GPCRs coupling to the same G protein in a single cell can elicit different biochemical or cellular responses (Harper et al., 1985; Steinberg and Brunton, 2001). A one-dimensional view of GPCR signal organization cannot readily account for such observations. At least two additional factors, compartmentalization of signaling proteins within membrane microdomains and preorganization of GPCR signaling units through interactions with anchoring and scaffolding proteins, appear to play important roles in GPCR signaling specificity.

Specificity arising within membrane microdomains. For most of the early years, GPCR signal transduction was conceptualized along the lines of a 'Brownian motion' model in which the random thermodynamic collision of signaling proteins within the plane of the plasma membrane was responsible for the flow of information from receptor to G protein to effector. Such a random process, however, would be energetically expensive for complex organisms that require rapidity and specificity of signaling function. Furthermore, mounting experimental evidence indicates that GPCRs, G proteins, and

effectors are not randomly distributed in the plasma membrane. Indeed, it has been suggested that GPCR signaling mainly occurs within specialized microdomains, implying that the efficiency and specificity of signal transduction are dictated by the stoichiometry of transducer elements within spatially discrete membrane regions (Neubig, 1994; Ostrom et al., 2000).

One of the most studied forms of membrane microdomain are regions of high density cholesterol, gangliosides and sphingolipids referred to as caveolae or lipid rafts (Galbiati et al., 2001). Many GPCRs have been shown to localize to these structures, often aided by C-terminal palmitoylation. A striking example of how localization of a GPCR within lipid microdomains dictates signal selectivity is the oxytocin receptor. When present in caveolae the receptor exerts a proliferative effect upon HEK293 cells through a Gq-mediated mechanism involving cross talk with epidermal growth factor (EGF) receptors that also concentrate in caveoli. Oxytocin-stimulated EGF receptor 'transactivation' is independent of phospholipase C, c-Src or phosphoinostide-3 kinase (PI3-K) activity. In contrast, activation of oxytocin receptors outside of rafts produces the exact opposite effect, an inhibition of cell proliferation that is Gi-, phospholipase C-, c-Src- and PI3-K-dependent (Rimoldi et al., 2003). In addition to lipid rafts, other regions of the plasma membrane where signaling proteins are aggregated, such as focal adhesion complexes and clathrin-coated pits, appear to serve as sites of GPCR signal integration and specificity.

Scaffolding and preorganization as determinants of signal specificity. It is now clear that the intracellular domains of GPCRs participate in numerous interactions with cellular proteins that serve to organize the partners in a signaling cascade (Brady and Limbird, 2002). In essence, these scaffolds assemble GPCRs, G proteins, effectors and downstream elements into prearranged 'solid-state' signaling devices that impose crucial spatial resolution and signaling compartmentalization on GPCR-mediated signaling systems. For example, β2-adrenergic receptors have a well documented association with plasma membrane AKAP (A kinase anchoring proteins) proteins (Malbon et al., 2004). AKAPs act as dynamic platforms that orchestrate the interactions of protein kinases, including tyrosine kinases, protein phosphatases, *e.g.* calcineurin, and cytoskeletal

elements with β2 receptors. Other preformed complexes between the β2 receptor and potential effectors have been reported, including association with the EGF receptor, a target for GPCR-stimulated 'transactivation' (Maudsley et al., 2000), and recently with the BKCa large conductance Ca^{2+}-dependent potassium channel (Liu et al., 2004)

Several PDZ domain-containing proteins, besides NHERF/EBP50, interact with the distal C-terminus of select GPCRs and direct the assembly of functional protein networks (Bockaert et al., 2003). This pre-organization of GPCRs into 'signalsomes' appears to be particularly prevalent within the central nervous system, where signaling efficiency and spatial constraint are at a premium. PSD-95, membrane-associated guanylate kinase inverted-2 (MAGI-2), SH3 multiple ankyrin domain-containing protein (Shank)/somatostatin receptor interacting protein (SSTRIP), Protein interacting with C kinase 1 (PICK-1), multi-PDZ domain protein 1 (MUPP1), and spinophillin contain between one and thirteen PDZ domains and all can associate with GPCRs . Their binding to receptors has been reported to modulate such diverse properties as receptor dimerization, subcellular localization, effector coupling, and trafficking.

Other GPCR protein-protein interactions appear to influence receptor localization and trafficking, or dictate the specificity of the GPCR-G protein-effector interaction (Bockaert et al., 2003). The Homer proteins, which are involved in the control of actin filament dynamics, interact with polyproline sequences found in mGluR1α, mGluR5 metabotropic glutamate receptor, Shank/SSTRIP, IP3 receptors, ryanodine receptors, and P/Q type calcium channels. Homer proteins function in the organization of postsynaptic glutaminergic sites, and excitation-dependent expression of Homer isoforms affects mGluR trafficking and targeting to axons and dendrites. The dopamine receptor-interacting protein of 78 kDa (DRIP-78) binds to a C-terminal hydrophobic motif in D1 dopamine receptors and controls post-translational processing of the receptor, while binding of the t-complex testis-expressed 1 (TcTex-1) protein to the C-terminus of rhodopsin targets the receptor to the rod outer segment. The angiotensin receptor-associated protein (ATRAP) binds to the C-terminus of the angiotensin AT1a receptor and modulates receptor coupling to phospholipase C and AngII-stimulated transcriptional activation and cell proliferation. Actin-binding protein 280 (ABP-280 or filamin A) interacts with the third intracellular (IC3) loop of the D2 and 3 dopamine receptors.

ABP-280 binding fosters D2 receptor clustering at the plasma membrane and enhances the ability of D2 receptors to inhibit adenylate cyclase. The 14-3-3 proteins, a family of at least seven acidic brain proteins bind that to phosphorylated serine/threonine motifs, interact with the third intracellular loops of α2 adrenergic and $GABA_BR1$ receptors and appear to regulate GPCR dimerization, activation of the Ras/Raf cascade, and the localization of regulator of G protein signaling (RGS) proteins. Collectively, these examples illustrate the extent to which scaffolding protein interactions preorganize GPCR signals and ensure signal fidelity.

THERAPEUTIC IMPLICATIONS

Pharmacologic agents acting via GPCRs have traditionally been classified as agonists, partial agonists, or antagonists based on the two state model of receptor function embodied in the ternary complex model. Agonists and partial agonists are assumed to differ only in intrinsic efficacy, the ability to stabilize a receptor in the R* conformation. The 'active' conformation of the receptor is presumed to be the same wherever the receptor is expressed and irrespective of the stimulus-response element being activated. However, as data continue to emerge indicating that GPCR signal transduction is both more diverse and more specific than originally imagined, these fundamental concepts of receptor biology are being refined. The existence of multiple 'active' receptor states, of receptor-receptor and receptor-scaffold interactions that modify receptor pharmacology, of possible 'G protein-independent' signaling, and of tissue selective preorganization of signals, presents the opportunity to develop drugs that induce only a subset of the GPCR response profile. At the same time, signaling complexity implies the existence of pitfalls arising from unintended drug action.

The potential therapeutic implications of agonist selective signal trafficking extend beyond regulation of receptor-G protein coupling. For example, ligands that selectively induce receptor internalization could be beneficial in treatment of tolerance (Yu et al., 1997). Similarly, selective GPCR internalization may prevent HIV-1 infection through chemokine receptor fusion. Ligands that cause internalization of CXCR4 (Amara et al., 1997) or CCR5 (Simmons et al., 1997) have been shown to protect against HIV-1 infection *in vitro*. Selective removal of chemokine receptors from the cell surface could

be superior to blocking chemokine receptor interaction with HIV viral coat proteins because it would prevent the possible rapid emergence of resistant HIV variants through therapeutic pressure and mutation (Domingo et al., 1997). Receptor dimerization may also generate therapeutic targets with unique pharmacology and signaling characteristics. Receptor dimers have been implicated in numerous areas including HIV-1 infection (Kuhmann et al., 2000) and the function of cannabinoid receptors (Mukhopadhyay et al., 2000), GABA-B receptors (Jones et al., 1998), adenosine A1 receptors (Ciruela et al., 1997), δ-opioid receptors (Cvejic and Devi, 1997), β_2-adrenoreceptors (Hebert et al., 1996), and calcium-sensing receptors (Bai et al., 1998). Drugs that selectively target unique ligand-binding pockets generated through dimerization may produce effects not associated with monomeric receptor signaling. Finally, the ability of ligands to selectively activate non-G protein-mediated signaling through GPCRs may prove therapeutically relevant. In this context, the finding that antagonist or inverse agonist ligands of the β2-adrenergic and AT1a receptors can stimulate MAP kinase signaling through β-arrestin-mediated pathways (Wei et al., 2003; Azzi et al., 2003) interjects a note of caution, in that antagonists that provide benefit in heart failure by antagonizing G protein activation could act counterproductively if they also stimulate pathways involved in cardiomyocyte hypertrophy and vascular smooth muscle hyperplasia.

The concept of agonist selective trafficking of receptor signaling has received much attention as it prompts the search for drugs that can change the quality as well as the quantity of efficacy (Kenakin, 2002). It is now clear that even the terms agonist and antagonist are strictly context-dependent. If a ligand can discriminate between multiple 'active' receptor conformations to preferentially activate a subset of effector pathways, then agonist efficacy needs to be defined in terms of the assay used to measure receptor activation. In the broadest sense, all ligands that productively engage a GPCR have the potential to be 'pluri-protean', acting as both agonist and antagonist depending on the signaling function and the nature of the cellular environment. In some cases it may be useful to reclassify compounds based on a full profile of stimulus-response coupling. Separating agonists in this manner could offer insights into preferred profiles of agonism as compounds progress from screening assays into therapeutically-oriented secondary assays. In situations where an original screening program was limited to measuring a

single signaling pathway, consideration should be given to reexamining the properties of some compounds that were initially disregarded on the basis of apparently poor efficacy. As ongoing work provides greater insights into the multitude of factors that give texture to GPCR signaling, the challenge will be to exploit the complex behavior of these receptors for therapeutic advantage while minimizing the pitfalls associated with too narrow a vision of receptor function.

CONCLUSIONS

For the majority of its experimental lifetime, information flow through GPCRs has been envisioned as unidirectional, *i.e*. changes in receptor conformation produced by agonist binding promote the transfer of information from outside the cell inwards. Recent experimentation, however, has demonstrated that receptor conformation is also controlled by protein-protein interactions occurring inside the cell. Receptor dimerization and interactions with scaffolding and signaling proteins can modify ligand selectivity and predetermine, from a menu of available options, which intracellular responses will predominate. In essence, the influences on receptor conformation are bi-directional; internal factors change the conformation of the receptor to reflect the status of the intracellular milieu, while extracellular factors, i.e. agonists, convey information to the cell about the external environment. This concept has critical implications for receptor theory.

If one accepts the premise that the association of GPCRs with intracellular proteins places a constraint on the array of 'active' conformations that the receptor can adopt, then even within a single cell there may exist different 'flavors' of the same receptor, prewired to produce specific responses to preferred ligands. In the limit case of one cell expressing multiple copies of the same receptor it seems unlikely that every copy of the receptor would be coupled to the same signal transduction machinery at all times. This is certainly true if the receptor is susceptible to G protein 'switching' induced by heterologus desensitization Daaka et al., 1997) or capable of signaling through β–arrestins (Luttrell and Lefkowitz, 2002). These various receptor 'flavors' could preferentially interact with different ligands, e.g. CRLR-RAMP1 recognizing calcitonin gene-related peptides and CRLR-RAMP2-RCP binding adrenomedullin (McLatchie et

al., 1998; Evans et al., 2000), or activate different downstream effectors in response to the same ligand. We have recently invoked the latter scenario with respect to tissue-specific differences in GnRH receptor signaling (Maudsley et al., 2004).

An interesting corollary of this postulate is that GPCRs may not be 'truly' promiscuous in the sense of a single receptor interacting with multiple effector pathways in a random manner. If GPCRs can be preassembled with their downstream transduction machinery, it may be biophysically more efficient to generate a variety of receptor 'flavors' that are hard-wired to specific transduction pathways than to switch a single receptor between different pathways. Such a model could accommodate experimentally observed promiscuity if the primary response pathway is defined as that mediated by the receptor 'flavor' with the highest affinity for a given agonist, whereas promiscuous coupling results from the activation of alternative receptor 'flavors' that have lower affinity for the ligand and therefore are only activated by higher agonist concentrations. Reversal of agonist efficacy could similarly result from altered ligand selectivity imposed from inside the cell through protein-protein interactions affecting ligand affinity. While selective examples of each of these phenomena exist, additional experimentation will be required to determine whether these mechanisms have broad applicability to models of GPCR signaling.

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