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Rich tapestry of GPCR signaling and regulatory mechanisms

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Running title: Diverse mechanisms of GPCR regulation

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Abstract

G protein-coupled receptors (GPCRs) are the largest family of signaling proteins and the most common therapeutic targets. In the last two decades, an impressive progress in the understanding of GPCR function has been achieved, largely driven by the idea of similarity of the molecular mechanisms underlying their signaling and regulation. However, recent comprehensive studies of signaling and trafficking of several GPCR subtypes, including endogenous M3 muscarinic and H1 histamine receptor and expressed cysteinyl leukotriene type 1 receptor in HEK293 cells, clearly demonstrate that each receptor is regulated by a unique set of molecular mechanisms involving different players. These data indicate that the "gold mine" of similarities is nearly exhausted, and that extrapolation from one receptor to another is as likely to be misleading as illuminating. Further progress in the field requires careful analysis of the regulation of individual GPCR subtypes in defined cellular context.

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Striking similarity between the signaling pathways that translate light captured by rhodopsin into the cGMP phosphodiesterase activity in photoreceptors and those converting hormonal activation of β2-adrenergic receptor (b2AR) into the adenylyl cyclase activity in other cells was noted in mid-eighties (Bitensky et al., 1984). However, the GPCR field as we know it was born after seminal elucidation of the b2AR structure, which clearly showed that rhodopsin and b2AR belong to the same protein family (Dixon et al., 1986). This discovery suggested that the mechanisms regulating rhodopsin activity, such as phosphorylation and arrestin binding, likely operate in the b2AR-driven signaling pathway. The idea proved remarkably fruitful: the first functional analog of rhodopsin kinase, β-adrenergic receptor kinase (βARK; now known as G protein-coupled receptor kinase 2, or GRK2) was identified the same year (Benovic et al., 1986). Subsequent elegant experiments demonstrated that phosphorylation alone does not fully account for b2AR desensitization, suggesting the role for an arrestin analog that binds non-visual GPCRs (Benovic et al., 1987), which was soon discovered and termed β-arrestin (now known as arrestin2 or β-arrestin1) (Lohse et al., 1990). The cloning of additional GPCRs (Frielle et al., 1987; Kobilka et al., 1987), GRKs (Benovic et al., 1989; Benovic and Gomez, 1993; Kunapuli and Benovic, 1993), and arrestins (Attramadal et al., 1992; Sterne-Marr et al., 1993) added further proof of sequence similarity between these proteins and their respective visual counterparts (Lorenz et al., 1991; Shinohara et al., 1987), reinforcing the view that most, if not all, GPCRs signal similarly and are controlled by the same regulatory mechanisms. So, when receptor-bound arrestins were found to act as adaptors linking the receptor to the components of the internalization machinery of the coated pit, clathrin (Goodman et al., 1996) and AP2 (Laporte et al., 1999), to mobilize and activate c-Src (Luttrell et al., 1999), and to scaffold kinase cascades activating JNK3 (McDonald et al., 2000) and ERK1/2 (Luttrell et al., 2001), it was implicitly

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assumed that these findings apply to pretty much all GPCRs. The data indicating that this is not necessarily the case were sometimes dismissed as inconsequential details. Partly due to this tradition of generalization, the beautiful demonstration of the dimeric nature of a small group of class C GPCRs (reviewed in (Pin et al., 2003)), along with evidence for dimerization of several class A receptors under certain circumstances, was interpreted by some as proof that all GPCRs exist as dimers, and that receptor dimers are necessary to interact with G proteins, arrestins, and other binding partners (e.g., see (Fotiadis et al., 2006)). Although rigorous experimental testing revealed serious limitations of this model ((Bayburt et al., 2007; Hanson et al., 2007b; James et al., 2006; Whorton et al., 2007), reviewed in (Gurevich and Gurevich, 2008a; Gurevich and Gurevich, 2008b)), in some ways this proved beneficial for the field, forcing us to see the potential problems with generalizations and pay close attention to the particulars of the regulation of individual receptors in defined cellular context.

The paper by Luo et al in this issue (Luo et al., 2008) is an excellent example of this type of study. The authors comprehensively explored the signaling by M3 muscarinic acetylcholine receptor endogenously expressed in HEK293 cells by knocking down individual regulatory proteins. Luo and colleagues found that GRK2, GRK3, and GRK6 significantly contribute to desensitization of the M3 receptor, whereas GRK5 does not. In addition, knockdown of either arrestin2 or arrestin3 increased M3-stimulated calcium response, implicating both subtypes in M3 desensitization. The authors confirmed earlier observations (Budd et al., 2000) that casein kinase-1γ (CKIγ) also participates in the suppression of M3-mediated calcium signaling. It is worth noting that the list of kinases other than GRKs that phosphorylate GPCRs and directly regulate their activity is growing. We can expect its further expansion as more receptor subtypes are carefully studied. The role of CKIγ in M3 receptor signaling is similar to the recently

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described key role of PKC phosphorylation in cysteinyl leukotriene type 1 receptor (CL1R) desensitization (Naik et al., 2005). However, the discovery of the crucial role of PKC in CL1R endocytosis (Naik et al., 2005), which was often thought to be mediated by GRK phosphorylation of the receptor and subsequent arrestin binding, reveals an additional rather unexpected role that phosphorylation of GPCRs by a variety of kinases might play. Interestingly, PKC-induced CL1R endocytosis is arrestin-independent, although CL1R can also internalize in arrestin-dependent manner (Naik et al., 2005). Although this aspect of GPCR trafficking is often overlooked, CL1R is not the only receptor that internalizes via more than one pathway: this phenomenon was reported with M2 muscrarinic acetylcholine receptor (Pals-Rylaarsdam et al., 1997) and several other GPCR subtypes (reviewed in (Gurevich and Gurevich, 2006b)). To summarize the study of Luo and colleagues, a large number of regulatory proteins, including three different GRKs, CKI□, and two non-visual arrestins, are required for the normal attenuation of calcium response to endogenous M3 receptor activation in HEK293 cells.

An important point highlighted in this work and several previous studies is the multi-functionality of GRKs and arrestins. Virtually every protein has multiple functions, all of which are indiscriminately suppressed by its knockdown. Even when a certain GRK or arrestin subtype is identified as a player in the regulation of a particular receptor, without direct evidence we cannot assume that the GRK in question acts via receptor phosphorylation, or that arrestin affects signaling via its binding to active GRK-phosphorylated receptor. GRKs carry a regulator of G protein signaling (RGS) domain on their N-terminus that binds active GTP-liganded □-subunits of Gq/11 (Carman et al., 1999). Thus, GRKs can inhibit the signaling of Gq/11-coupled receptors, such as M3, via at least two distinct mechanisms: by phosphorylating the receptor to promote arrestin-mediated uncoupling from cognate G proteins (Gurevich and Gurevich, 2004),

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and by sequestering activated α-subunits of Gq and G11 (Carman et al., 1999). To elucidate the actual mechanism of GRK2-mediated inhibition of M3 receptor signaling, the authors used precisely targeted tools: GRK2 mutants devoid of either kinase activity or the ability to bind Gq/11 α-subunits. Since kinase-dead GRK2-K220R turned out to be as effective as wild type GRK2, whereas both mutants defective in Gαq binding had no effect on M3 receptor signaling, the data clearly demonstrate that GRK2 largely mediates M3 desensitization via sequestration of Gαq/11 (Luo et al., 2008). In addition to sequestering and silencing Gαq/11 (Carman et al., 1999; Iwata et al., 2005; Luo et al., 2008), GRK2 binds G$\beta\gamma$ dimers (Pitcher et al., 1992), blunts ERK1/2 activation by binding MEK1 (Jiménez-Sainz et al., 2006), and phosphorylates quite a few non-GPCR substrates, such as tubulin (Carman et al., 1998) and ezrin (Cant and Pitcher, 2005). In addition, GRKs 2 and 5 phosphorylate several isoforms of synuclein (Pronin et al., 2000). GRKs can phosphorylate these proteins independently of receptor activation in vitro. However, GRK interactions with phospholipids and G$\beta\gamma$, which are promoted by GRK recruitment to active GPCRs in the cell, enhance phosphorylation of non-receptor substrates. In most cases, we do not know whether phosphorylation of these substrates is actually affected by receptor activation. A well described example of such a link is the phosphorylation of ezrin by GRK2, which is a necessary step in the receptor activation-dependent reorganization of the actin cytoskeleton (Cant and Pitcher, 2005).

In terms of known multi-functionality, arrestins are way ahead of GRKs, interacting with an incredible variety of signaling proteins (Gurevich and Gurevich, 2006a; Xiao et al., 2007). Multiple partners preferentially interact with receptor-bound arrestins (Gurevich and Gurevich, 2003; Lefkowitz and Shenoy, 2005), some (e.g., microtubules (Hanson et al., 2007a) and calmodulin (Wu et al., 2006)) exclusively bind free arrestins because their interaction sites

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overlap with that of the receptor (Hanson et al., 2006; Vishnivetskiy et al., 2004), whereas others simply prefer arrestin in its free "inactive" conformation (Song et al., 2006). Bound arrestin not only covers the cytoplasmic tip of the receptor, "crowding out" G proteins (Krupnick et al., 1997), but often initiates the second round of signaling (Gurevich and Gurevich, 2003; Lefkowitz and Shenoy, 2005), serving as a scaffold for MAP kinase cascades (Luttrell et al., 2001; McDonald et al., 2000). GPCR activation can be translated into ERK1/2 phosphorylation via distinct mechanisms mediated by G proteins or arrestins. After similar stories emerged from studies with angiotensin II (Ahn et al., 2004), □2-adrenergic (Shenoy et al., 2006) and parathyroid hormone (Gesty-Palmer et al., 2006) receptors, a novel paradigm was proposed that G-protein-mediated ERK phosphorylation is very transient, whereas arrestins mediate sustained ERK activation. The study of the M3 receptor shows that this not always the case: Luo et al (Luo et al., 2008) demonstrated that non-visual arrestins are key players in M3 receptor desensitization, but detected no arrestin-dependent ERK activation via endogenous M3 receptor. Interestingly, in this case G protein-mediated ERK phosphorylation was sustained for up to 60 min, and was further enhanced and prolonged by knockdown of GRK2 and arrestins. Obviously, interactions of GRKs and arrestins with multiple non-GPCR partners can significantly affect receptor signaling. As beautifully illustrated by the work of Luo et al (Luo et al., 2008), the actions of GRK2 in HEK293 cells on both the M3 receptor-stimulated calcium mobilization and ERK1/2 activation are mediated by interactions with non-receptor partners, and neither involves receptor phosphorylation.

M3 is the third receptor comprehensively studied by Dr. Benovic and colleagues in HEK293 cells (Iwata et al., 2005; Luo et al., 2008; Naik et al., 2005). The regulation of b2AR (Violin et al., 2008; Violin et al., 2006b) and angiotensin II type 1A receptor (AT1AR) (Violin et al.,

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2006a) in these cells was extensively studied by Dr. Lefkowitz group. It is important to note that the only unifying conclusion of these studies is that each GPCR subtype has a unique pattern of regulation. M3 receptor is desensitized via phosphorylation by GRKs 3, 6, and CKI (Luo et al., 2008) and GRK2-mediated sequestration of Gq; histamine H1 receptor is primarily desensitized by GRK2 via both phosphorylation and Gq binding (Iwata et al., 2005); AT1AR is desensitized by GRK2 (Violin et al., 2006a); b2AR was found to be regulated by GRKs 2 and 6 when arrestin recruitment was used as a readout (Violin et al., 2006b), but predominantly by GRK6 when cAMP response was measured instead (Violin et al., 2008); whereas desensitization as well as arrestin-independent internalization of CL1R requires receptor phosphorylation by PKC (Naik et al., 2005). Unexpectedly, arrestin recruitment to b2AR in HEK293 cells does not appear to correlate with the bulk of receptor phosphorylation, suggesting that as far as arrestin is concerned, GRK phosphorylation sites are not equivalent (Violin et al., 2006b). Remarkably, GRK5, which is one of the two most abundant GRKs in HEK293 cells, does not appear to participate in the regulation of any of these receptors. Interestingly, GRK3 was found to play more prominent role in b2AR desensitization in U2-OS osteosarcoma cells that express higher levels of this isoform than in HEK293 cells (Violin et al., 2006b). These data bring up an important question of receptor specificity of GRK isoforms, which is usually considered only in terms of preferential phosphorylation of certain GPCR subtypes by a particular GRK. However, because GRKs have functional capabilities that do not involve the kinase activity, this issue is much more complex. For example, RGS domain of GRKs 2 and 3 sequesters GTP-liganded Gq/11, but not -subunits of other G proteins. Therefore, this mechanism of GRK2/3 action is specific for Gq/11-dependent signaling pathways. The same two GRKs bind -dimers suppressing -mediated signal transduction often important in signaling mediated by a different

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group of G proteins, Gi/o subfamily. In addition, the ability of GRK2 to inhibit ERK1/2 activation by MEK1 makes this mechanism specific for the pathways that involve the MEK1-ERK1/2 module. In most studies, in contrast to the work of Luo et al (Luo et al., 2008), the actual mechanism of GRK-dependent suppression of receptor signaling was not determined.

In mammals, four ubiquitously expressed GRKs (GRKs 2,3,5, and 6), as well as the more restricted GRK4, are available to regulate more than 700 GPCR subtypes. Obviously, 1:1 specificity for receptors is out of the question. However, it does not mean that GRK isoforms are simply redundant, e.g., can regulate any GPCR in the same manner. *In vitro* experiments using GRK overexpression often show that many GRK isoforms are able to promote desensitization and trafficking of various GPCRs. However, *in vivo* studies with knockout and transgenic mice provided evidence for unexpectedly strict receptor specificity of different GRKs. For example, the elimination of GRK6 causes behavioral supersensitivity to dopaminergic stimulation (Gainetdinov et al., 2003), whereas knockout of its closest relative GRK5 does not alter dopaminergic signaling (Gainetdinov et al., 1999). Instead, the loss of GRK5 enhances central responses to muscarinic stimulation (hypothermia, tremor, salivation, and locomotion) without affecting responsiveness to the □-opioid or 5-HT1A receptor stimulation (Gainetdinov et al., 1999). Mice lacking GRK3, 5, or 6 have relatively mild phenotypes, indicating that to a certain extent the remaining GRKs can take over the functions of the missing isoform. However, the fact that knockout of GRK2 is embryonic lethal (Jaber et al., 1996) proves that it has functions that cannot be performed by any other member of the family. At the same time, knockout of GRK3, the isoform remarkably similar to GRK2 structurally and biochemically (Arriza et al., 1992; Willets et al., 2003), produces only very mild neuronal phenotype (Gainetdinov et al., 2004). In some cases, apparent receptor specificity is based on specific cellular complement of GRK

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isoforms. For example, the loss of the odorant receptor desensitization in GRK3 knockout mice (Peppel et al., 1997) is due to the fact that GRK3 is the major, if not the only, isoform expressed in the olfactory epithelium. Nonetheless, even when multiple GRKs are expressed in the same cell, they often only regulate specific receptors and/or regulate the same GPCR via distinct non-overlapping mechanisms. For example, GRKs 2 and 3 are expressed in cardiac myocytes at similar levels, but GRK2 is primarily responsible for regulation of the β-adrenergic and angiotensin receptors (Vinge et al., 2007). In contrast, GRK3 does not seem to regulate β-adrenergic signaling, but appears to control α1-adrenergic and endothelin receptors in these cells (Eckhart et al., 2000; Vinge et al., 2007). This receptor specificity of GRKs 2 and 3 defines the biological role of each isoform in different aspects of heart function: GRK2 is the key player in the heart development and heart failure (Hansen et al., 2006; Jaber et al., 1996), whereas GRK3 is important for the control of the cardiac growth and hypertrophy (Vinge et al., 2007; Vinge et al., 2008). The work by Dr. Benovic's group lends further support for the idea that receptors are preferentially regulated by specific GRK isoforms. Moreover, when multiple GRKs regulate signaling by the same receptor, functional consequences differ depending on the isoform involved. Luo at al (Luo et al., 2008) found that knockdown of GRK2, 3, or 6 similarly enhances calcium mobilization via M3 receptor, whereas ERK activation by the same receptor was not affected by the GRK3 knockdown. It is remarkable that even though GRK2 and GRK5 are the two major isoforms in HEK293 cells, one regulates M3 muscarinic receptor without actually phosphorylating it, whereas the other does not affect it at all. These findings clearly show that when GRKs are expressed at physiological levels, different isoforms demonstrate pronounced receptor specificity. It is also clear that virtually every cell expresses multiple GRKs and many

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GPCRs. Therefore, the mere fact of coexpression does not mean that specific GRK isoform is in any way involved in the regulation of a particular receptor.

The situation with arrestins is even more intriguing, since there are only two ubiquitous isoforms, arrestin2 and 3, each represented by two splice variants (Sterne-Marr et al., 1993). In some tissues, particularly in the brain, the concentration of arrestin2 is many times higher than that of arrestin3, and this difference becomes more dramatic during development (Ahmed et al., 2008a; Ahmed et al., 2008b; Gurevich et al., 2002; Gurevich et al., 2004). The knockout of arrestin2 causes slightly increased responsiveness to β-adrenergic stimulation in the heart (Conner et al., 1997) and no enhanced behavioral responses to dopaminergic or μ-opioid drugs (Gainetdinov et al., 2004). In contrast, the ablation of arrestin3 elevates antinociceptive and rewarding effects of morphine, reduces tolerance to morphine, and increases μ-opioid receptor coupling to G proteins (Bohn et al., 2000; Bohn et al., 2003; Bohn et al., 1999). In the study of Luo et al (Luo et al., 2008), knockdown of either arrestin enhanced carbachol-induced calcium mobilization and ERK phosphorylation, although only the knockdown of arrestin3 resulted in prolonged ERK activation. Thus, the two non-visual arrestins are certainly non-redundant. Strong evidence of receptor and functional specificity of different arrestins and GRKs *in vitro* and *in vivo* is rapidly accumulating. It is becoming increasingly clear that, in addition to the nature of the receptor, many other factors contribute to the functional performance of individual arrestins and GRKs. Relative intracellular concentrations and the complement of arrestin and GRK isoforms in the cell, the subcellular distribution of the receptor and particular arrestins and GRKs, as well as the expression levels other signaling proteins all play a role. The precise experimental elucidation of the functional repertoire of each GRK and arrestin will significantly contribute to our ability to unravel the exceedingly complex cellular signaling network.

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In the last two decades, the key molecular mechanisms of GPCR signaling and its regulation have been elucidated. In the process, we learned that there is no such thing as a generic receptor, generic GRK, or a generic cell. The evolution endowed mammals with ~1,000 different GPCRs (Rompler et al., 2007) that are phosphorylated by seven GRKs (Moore et al., 2007) and a number of other kinases (Budd et al., 2000; Luo et al., 2008; Naik et al., 2005) and interact with four arrestin subtypes (Gurevich and Gurevich, 2006b). Each tissue and cell has a unique complement of receptors (Penn et al., 2001), GRKs and arrestins (Ahmed et al., 2008a; Ahmed et al., 2008b; Bychkov et al., 2008; Gurevich et al., 2002; Penn et al., 2001; Violin et al., 2006b) that changes, sometimes quite dramatically, during development (Gurevich et al., 2002; Gurevich et al., 2004), disease (Ahmed et al., 2008a; Bychkov et al., 2008), and drug treatment (Ahmed et al., 2008b). To make matters even more complicated, phosphorylation of the same receptor at different sites (Jones and Hinkle, 2008; Key et al., 2003; Lee et al., 2000; Pals-Rylaarsdam et al., 1997), by different GRKs (Kim et al., 2005; Luo et al., 2008; Ren et al., 2005; Violin et al., 2006b), or even by the same GRK to different levels (Vishnivetskiy et al., 2007) generates functionally distinct receptor species that bind arrestins with different biological consequences. Apparently, thousands of distinct patterns of signaling and regulation generated by this variety are necessary for survival. We have a huge task of elucidating these patterns for each receptor in every cell type where it is endogenously expressed to understand the biological significance of each thread in this incredibly rich tapestry of signaling regulation.

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