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G protein-coupled receptor kinases: more than just kinases and not only for GPCRs

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Abstract

G protein-coupled receptor (GPCR) kinases (GRKs) are best known for their role in homologous desensitization of GPCRs. GRKs phosphorylate activated receptors and promote high affinity binding of arrestins, which precludes G protein coupling. GRKs have a multidomain structure, with the kinase domain inserted into a loop of a regulator of G protein signaling homology domain. Unlike many other kinases, GRKs do not need to be phosphorylated in their activation loop to achieve an activated state. Instead, they are directly activated by docking with active GPCRs. In this manner they are able to selectively phosphorylate Ser/Thr residues on only the activated form of the receptor, unlike related kinases such as protein kinase A. GRKs also phosphorylate a variety of non-GPCR substrates and regulate several signaling pathways via direct interactions with other proteins in a phosphorylation-independent manner. Multiple GRK subtypes are present in virtually every animal cell, with the highest expression levels found in neurons, with their extensive and complex signal regulation. Insufficient or excessive GRK activity was implicated in a variety of human disorders, ranging from heart failure to depression to Parkinson's disease. As key regulators of GPCR-dependent and -independent signaling pathways, GRKs are emerging drug targets and promising molecular tools for therapy. Targeted modulation of expression and/or of activity of several GRK isoforms for therapeutic purposes was recently validated in cardiac disorders and Parkinson's disease.

Keywords

G protein-coupled receptors; G protein-coupled receptor kinases; signaling; regulation; phosphorylation; G proteins; regulator of G protein signaling

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Conflict of Interest Statement

The authors declare that there are no conflicts of interests.

1. Introduction

Signaling via G protein-coupled receptors (GPCR) is terminated by a remarkably uniform two-step mechanism: a GPCR kinase (GRK) phosphorylates the active receptor, converting it into a target for high affinity binding of arrestin. Bound arrestin shields the cytoplasmic surface of the receptor, precluding G protein binding and activation (Wilden, 1995; Krupnick et al., 1997).

Phosphorylation of rhodopsin, a prototypical GPCR, upon its activation by light was first described in 1972 (Bownds et al., 1972; Kühn and Dreyer, 1972). Soon thereafter "opsin kinase" (modern name GRK1[1]), which selectively phosphorylates active rhodopsin, was identified (Weller et al., 1975). The first clear evidence that rhodopsin phosphorylation is necessary for its rapid deactivation was presented in 1980 and led to the hypothesis that this mechanism may also regulate hormone receptors (Liebman and Pugh, 1980). Within a few years, this idea was confirmed for β_2-adrenergic receptor (β2AR) (Stadel et al., 1983; Sibley et al., 1985) and later for many others (reviewed in (Carman and Benovic, 1998)). The demonstration of sequence similarity between the β2AR and rhodopsin in 1986 (Dixon et al., 1986) led to the recognition of the family of G protein-coupled receptors (GPCRs), of which rhodopsin is a founding member. Also in 1986, a kinase that could phosphorylate activated β-adrenergic receptors (βARK; modern name GRK2) was identified (Benovic et al., 1986a). This enzyme could also phosphorylate rhodopsin in a light-dependent manner (Benovic et al., 1986b). Phosphorylation of rhodopsin facilitates the binding of another protein termed arrestin (called 48-kDa protein at the time), which physically blocks further signaling by the receptor to heterotrimeric G proteins (Wilden et al., 1986). Demonstration that desensitization of the β2AR requires a homolog of arrestin (Benovic et al., 1987) firmly established the paradigm of two-step GPCR inactivation, which was later shown to apply to the majority of GPCRs (Carman and Benovic, 1998; Gurevich and Gurevich, 2004, 2006b). The cloning of GRK2 in 1989 suggested that it belongs to a distinct lineage of eukaryotic Ser/Thr protein kinases (Benovic et al., 1989b) that are a subclass of the AGC kinase group (Manning et al., 2002). In rapid succession, the members of this family expanded to include βARK (GRK3) (Benovic et al., 1991), GRK4 (Ambrose et al., 1992), GRK5 (Kunapuli and Benovic, 1993), and GRK6 (Benovic and Gomez, 1993). Cone specific GRK7 (Hisatomi et al., 1998; Weiss et al., 1998) completed the set of vertebrate GRKs.

The expression of mammalian GRK1 and GRK7 is largely limited to vertebrate rod and cone photoreceptors although both are also present in pinealocytes (Somers and Klein, 1984; Zhao et al., 1997; Zhao et al., 1999; Pugh and Lamb, 2000). Virtually every mammalian cell expresses several isoforms of non-visual GRKs from early embryonic development. GRK4 is expressed at high levels only in testis (Premont et al., 1996). In addition, GRK4 expression was detected in proximal tubule cells in kidneys, where GRK4α and GRK4γ variants reportedly regulate the signaling of D1 and D3 dopamine receptors (Felder et al., 2002; Villar et al., 2009). GRK4 is also expressed in the brain (Sallese et al., 2000b) and uterus myometrium (Brenninkmeijer et al., 1999). In the rat brain, four GRK isoforms, GRKs 2, 3, 5, and 6, are found as early as embryonic day 14 (Gurevich et al., 2004). Unfortunately, the information about the cell-specific expression of GRK isoforms is

[1]In this review, systematic names of vertebrate GRK proteins are used: GRK1 (historic names opsin kinase, rhodopsin kinase), GRK2 (historic name β-adrenorenergic receptor kinase 1), GRK3 (historic name β-adrenorenergic receptor kinase 2), GRK4, GRK5, GRK6, GRK7 (historic name cone kinase). The proteins and gene symbols for human and other mammalian and non-mammalian vertebrate GRKs are used in accordance with published guidelines [H.M. Wain, E.A. Bruford, R.C. Lovering, M.J. Lusha, M.W. Wright and S. Povey, Guidelines for Human Gene Nomenclature *Genomics* **79** (2002), pp. 464-470, Suggested Xenopus Gene Name Guidelines (2005), http://www.xenbase.org/gene/static/geneNomenclature.jsp, Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat (2010), http://www.informatics.jax.org/mgihome/nomen/gene.shtml, ZFIN Zebrafish Nomenclature Guidelines (2011), https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines.]

limited. We mostly know their distribution at the tissue level. The cellular complement of GRK isoforms may prove to be the most important determinant of specificity in GRK function. For example, both GRK1 and GRK2 efficiently phosphorylate light-activated rhodopsin, but GRK2 does not perform this function in GRK1 knockout mice.

The importance of the GRK-mediated signal shutoff is best illustrated in the visual system, where the lack of GRK1 or sites for GRK phosphorylation on rhodopsin leads to the loss of photoresponses, photoreceptor degeneration, and blindness in mice and night blindness in humans (Chen et al., 1995b; Yamamoto et al., 1997; Khani et al., 1998; Chen et al., 1999b; Zhang et al., 2005; Hayashi et al., 2007; Song et al., 2009; Fan et al., 2010). In other cell types, the results are not as dramatic, except in development. In *Drosophila*, Gprk2[2], an ortholog of GRK4/5/6, is required for wing morphogenesis (Molnar et al., 2007b), egg morphogenesis, and embryogenesis (Schneider and Spradling, 1997). Knockdown of Grk2 in zebrafish embryos induces early developmental arrest (Jiang et al., 2009), and knockout of GRK2 in mice is embryonic lethal due to abnormal formation of the heart (Jaber et al., 1996;). This lethality stems from general, albeit undefined, role of GRK2 in embryogenesis, rather than specific role in the heart development, because mice with GRK2 ablation specific to the cardiac myocytes develop normally (Matkovich et al., 2006).

We know about structure and function of GRKs a lot less than these proteins deserve, considering that GRKs critically influence the function of most GPCRs, which are the targets of a large percentage of clinically used drugs (Gruber et al., 2010). Many issues are far from resolved. GRK specificity towards particular receptor subtypes is one important unanswered question. As mammals have only five non-visual GRKs and >800 GPCRs (Gruber et al., 2010), there are hundreds of GPCRs per GRK. It follows that each GRK must have the ability to phosphorylate many different receptors. However, neither the level of receptor specificity nor actual preference for particular GPCRs of non-visual GRKs is clear. We also need to fully describe how active GPCRs activate GRKs, which would be greatly facilitated by a structure of a receptor-GRK complex. This would define the full receptor footprint on the GRK and provide greater insight into the mechanism of kinase activation. GRKs phosphorylate many non-GPCR substrates, but it remains unknown whether proteins other than GPCRs can activate GRKs.

GRKs 2 and 5 have long been considered promising therapeutic targets for cardiac diseases (Penela et al., 2006). However, there has been little research regarding their value as therapeutic targets for other conditions. We believe that GRKs, by virtue of their regulatory nature, hold a great promise for therapy of disorders involving an imbalance in GPCR signaling. However, a better understanding of their structure and function is a prerequisite for successful therapeutic intervention.

2. Structural organization of GRKs

Based on sequence similarity and gene structure, vertebrate GRKs are classified into three subfamilies: GRK1 comprising GRK1 (rhodopsin kinase) and GRK7 (cone kinase), GRK2 comprising GRK2 and 3, and GRK4 comprising GRK4, 5, and 6 (Premont et al., 1999). All GRKs are multi-domain proteins (Fig. 1) consisting of ~25-residue N-terminal region unique to the GRK family of kinases, followed by the regulator of G protein signaling (RGS) homology domain (RH) (Siderovski et al., 1996), and a Ser/Thr protein kinase domain (KD) that belongs to the AGC kinase family (Fig. 1). This ~500–520 residue

[2]For invertebrate GRKs, we use the names employed in the original publications, with the indication of the vertebrate orthologs, since there are no strict guidelines for the denotations of protein abbreviations for invertebrates [Genetic nomenclature for Drosophila melanogaster (2011), http://flybase.org/static_pages/docs/nomenclature/nomenclature3.html.].

assembly is shared by all GRKs. The C-termini of GRKs contain structural elements responsible for their membrane targeting: GRK1 and 7 carry short C-terminal prenylation sequences, GRK2 and 3 contain pleckstrin homology (PH) domains that interact with G protein βγ (Gβγ) subunits (Pitcher et al., 1992; Koch et al., 1993; DebBurman et al., 1996), GRK4 and 6 carry palmitoylation sites (Stoffel et al., 1994; Premont et al., 1996) as well as lipid-binding positively charged elements (Jiang et al., 2007), whereas GRK5 relies on positively charged lipid-binding elements (Pitcher et al., 1996; Thiyagarajan et al., 2004). This domain composition correlates with the degree of sequence similarity in the shared domains. Crystal structures of one representative from each subfamily were solved: GRK2 alone (Lodowski et al., 2005), in complex with Gβγ-subunit (Lodowski et al., 2003), and with both $G\alpha_q$ and Gβγ subunits of the heterotrimeric G protein (Tesmer et al., 2005), as well as GRK6 (Lodowski et al., 2006), and GRK1 (Singh et al., 2008). The structures suggest that GRK RH/KD core appeared as the result of the insertion of KD into α9-α10 loop of an ancestral RH domain (Lodowski et al., 2003; Lodowski et al., 2006), whereupon different family members acquired various additional structural elements. A feature distinguishing most of these GRK structures from other AGC kinases is that the two lobes of the KD are in an “open” orientation and “nucleotide gate” region in the carboxyl-terminal tail (C-tail) of the kinase is disordered regardless of the presence of an ATP analogue or G protein subunits (Lodowski et al., 2006), which is atypical for an AGC kinase. This indicates that the kinase requires an induced rearrangement to become active, which is apparently provided by docking with active GPCRs, consistent with biochemical data (Palczewski et al., 1991; Chen et al., 1993). In one recent structure of GRK6, the kinase domain does adopt a relatively closed conformation, and the unique N-terminus and C-tail region coalesce with the small lobe of the kinase domain to form what is expected to be a receptor docking site (see below) (Boguth et al., 2010). This structure reveals an extensive flat surface adjacent to the docking site with abundant positive charges that likely faces the membrane. This conclusion is supported by the finding that the lipid anchors of G protein subunits bound to GRK2, although disordered in crystal structures, are also generally localized the same side of the kinase domain (Lodowski et al., 2003; Tesmer et al., 2005). The GRK N-terminus, as well as several receptor-facing residues in the kinase domain, seem to mediate allosteric activation of GRKs by the active GPCRs (reviewed in (Huang and Tesmer, 2011)). The binding of other proteins, such as recoverin, to the N-terminus inhibits receptor-dependent kinase activation (Higgins et al., 2006).

The *GRK1* and *7*genes in humans contain exons, the genes of *GRK2* and *3* have 21 exons, and members of the *GRK4* subfamily have 16 exons. Despite multi-exon structure of all *GRK* genes, alternative splicing was reported only for *GRK4* subfamily transcripts (Fig. 1). Four splice variants of human GRK4 were described: GRK4α(578 amino acids) containing all 16 exons, GRK4β (546 amino acids) lacking in-frame exon XV, GRK4γ (532 amino acids) lacking in-frame exon II, and GRK4δ (500 amino acids) lacking both N-terminal exon II and C-terminal exon XV (Premont et al., 1996; Sallese et al., 1997; Premont et al., 1999). However, orthologous mouse GRK4 protein appears to exist as only a single 574 amino acid variant (Premont et al., 1999). Interestingly, the shorter 545 amino acid splice variant of GRK4 found in rat, which lacks exon VI, has no human equivalent (Virlon et al., 1998). The longest rat (Virlon et al., 1998) and mouse (Premont et al., 1999) GRK4 variants display 76% and 77% amino acid sequence identity with human GRK4α. Human *GRK6* gene generates three alternative splice variants: GRK6A, B, and C. The GRK6B is the longest, with 589 amino acids. When exon XVI starts two nucleotides downstream from that in GRK6B, resulting in a frame shift, GRK6A (576 amino acids), with a different C-tail sequence is generated. The shortest isoform GRK6C (560 amino acids) is generated by utilizing an alternative exon XVI upstream, which encodes only a single amino acid before the stop codon. In contrast to GRK4, these splice variants are conserved in mouse (Premont et al., 1999) and rat (Firsov and Elalouf, 1997).

3. Subcellular targeting of GRK isoforms

The various GRK subfamilies employ several distinct mechanisms that bring them to or retain them at the membrane, where their integral membrane substrates GPCRs are found (Fig. 1). Visual subtypes have characteristic CaaX motif on the C-terminus for prenylation: GRK1 is farnesylated (Inglese et al., 1992a), whereas GRK7 is geranylgeranylated (Hisatomi et al., 1998). The association of GRKs 1 and 7 with the membrane is mediated by C-terminal prenylation. Therefore, visual GRKs "search" for active rhodopsin and cone opsins via diffusion in two dimensions on the membrane, which is much faster than 3D diffusion. This is important for the sub-second shutoff kinetics of light-induced signaling in photoreceptors (Krispel et al., 2006). Although GRK1, which is farnesylated, is rather loosely associated with the membrane of rod outer segments (ROS) in the dark (Kühn, 1978; Anant and Fung, 1992), and its membrane association is enhanced by light exposure (Kühn, 1978), farnesylation is important for its activity (Inglese et al., 1992b). This importance is further underscored by delayed photoresponse recovery due to impaired GRK1 transport to the rod outer segment membranes in mice lacking prenyl binding protein PrBP/δ (Zhang et al., 2007). Membrane targeting of GRKs 2 and 3 is signaling-dependent, because Gβγ subunits generated by the active receptor recruit these two GRKs to the locale of activated GPCRs, facilitating GPCR phosphorylation (Haga and Haga, 1992; Pitcher et al., 1992; Pitcher et al., 1995; Li et al., 2003). Palmitoylation of GRK4 and 6 (Stoffel et al., 1994; Premont et al., 1996; Loudon and Benovic, 1997) and lipid binding by GRK5 and GRK6 (Pitcher et al., 1996; Loudon and Benovic, 1997; Stoffel et al., 1998; Thiyagarajan et al., 2004; Tran et al., 2007) enhance affinity for the lipid bilayer and the kinase activity. The C-terminus of GRK6A splice variant contains multiple elements promoting or inhibiting membrane localization, including palmitoylation sites (Stoffel et al., 1994), a lipid-binding amphipathic helix (Jiang et al., 2007), and acidic residues that inhibit membrane localization (Vatter et al., 2005; Jiang et al., 2007). A non-palmitoylated form of GRK6A is not localized to the plasma membrane and instead is detected in the cytoplasm and the nucleus (Jiang et al., 2007). Interestingly, GRK6B and GRK6C splice variants lacking palmitoylation sites still strongly localize to the plasma membrane in cultured cells (Vatter et al., 2005). Apparently, the GRK6A C terminus contains a string of acidic amino acids that negatively regulate membrane localization of the protein, since mutation of these residues to neutral or basic rescues membrane localization of non-palmitoylated GRK6A (Jiang et al., 2007). Thus, GRK6B and GRK6C splicing variants lacking C tail of GRK6A (GRK6B due to a frame shift, GRK6C due to early translation termination) are able to localize to the membrane without the need for palmitoylation. These data suggest that palmitoylation works is specific structural context and may not the dominant mechanism of membrane localization of GRK6A, consistent with the recent crystal structure of GRK6A in which the palmitoylation sites were shown to be relatively distant from the expected membrane-binding surface (Boguth et al., 2010). GRK5 and GRK6A have been detected in the nucleus (Johnson et al., 2004; Jiang et al., 2007; Martini et al., 2008), and GRK5 contains a sequence that mediates both nuclear localization and DNA binding (Johnson et al., 2004), suggesting that these kinases might participate in the regulation of transcription via epigenetic mechanisms. Indeed, increased accumulation of GRK5 in the nuclei of cardiac myocytes has been shown to promote cardiac hypertrophy and early heart failure due to GRK5 acting as a class II histone deacetylase kinase (Martini et al., 2008).

Subcellular localization of GRKs has chiefly been described in cultured cells, with attention focused on mechanisms of their membrane recruitment. However, many cells are highly compartmentalized and contain multiple types of specialized membranes absent in cultured cells. This is particularly obvious in neurons, which arguably perform more signaling than any other cell type. Mature neurons have very sophisticated shape with large multi-branched dendritic trees and long axons that often terminate at multiple pre-synapses, transmitting the

signal to many post-synaptic cells. Importantly, critical role of complex shape of the cytoplasm in the kinetics and reliability of signaling was recently demonstrated in rod photoreceptors (Bisegna et al., 2008; Caruso et al., 2011). Obviously, it is of prime interest how GRK isoforms are targeted to specialized membrane compartments in specific cell types. Differential subcellular targeting might contribute to functional specificity of GRK isoforms, even without receptor preference at the molecular level. In the brain, GRK2 and 3 isoforms show somewhat different subcellular distribution, with GRK3 being more membrane-associated than GRK2 (Ahmed et al., 2007; Bychkov et al., 2008), although they behave similarly in HEK293 cells. GRK5 and 6 preferentially localize to synaptic membranes (Ahmed et al., 2007; Ahmed et al., 2010; Bychkov et al., 2011). Currently, there are no known mechanisms that would ensure specific localization to the synaptic as opposed to the plasma membrane. These data suggest that there are cell type-specific mechanisms targeting GRKs to different compartments that need to be investigated in relevant cells, such as neurons, *in vivo*.

4. Mechanism of GRK activation by active GPCRs

The ability to phosphorylate active GPCRs was the first GRK function to be discovered. Receptor phosphorylation by itself can decrease G protein coupling (Wilden, 1995) and enables high-affinity binding of arrestin, which stops G protein-mediated signaling by blocking the cytoplasmic surface of the receptors (Krupnick et al., 1997). The most striking feature distinguishing GRKs from other kinases is that their activity depends on the functional state of the target: GRKs effectively phosphorylate active GPCRs. However, they are clearly capable of phosphorylating other targets at the membrane in response to receptor activation, as exemplified by the so-called “high-gain” phosphorylation of many more rhodopsin molecules that were light-activated (Binder et al., 1990; Binder et al., 1996), as well as phosphorylation of rhodopsin upon activation of transgenically co-expressed cone opsin with distinct spectral sensitivity in rods, and *vice versa* (Shi et al., 2005). An increase of the availability of receptor phosphorylation sites upon their activation does not appear to play a role, because active GPCRs can enhance GRK phosphorylation of exogenous peptide substrates, indicating that docking with the active receptor directly activates the GRK (Palczewski et al., 1991; Chen et al., 1993). This receptor-dependent activation functions with non-cognate pairs, as demonstrated by the fact that GRK2 robustly phosphorylates rhodopsin in strictly light-dependent fashion (Benovic et al., 1986b). A shared activation mechanism of GRKs relying on common structural features presented by active GPCRs thus seems to allow relatively few GRKs to phosphorylate hundreds of structurally different receptors (Palczewski, 1997). Interestingly, GRK4 is the only GRK isoforms (GRK4α splice variant) reported to be constitutively active and capable of phosphorylating unstimulated GPCRs (Ménard et al., 1996; Rankin et al., 2006).

Current ideas about the mechanism of activation of GRKs has been recently reviewed (Huang and Tesmer, 2011), and thus will only be briefly summarized here. A recent structure of GRK6 revealed for the first time a GRK in a conformation very similar to those of other activated AGC kinases (Boguth et al., 2010). In this structure, the 25 N-terminal residues are ordered and within this span form a single α helix that is engaged in extensive interactions with both the small lobe and the C-tail of the kinase domain. These interactions are not possible when GRK6 is in a more open conformation (Lodowski et al., 2006). The residues involved in the contacts between the N-terminal helix and the rest of the kinase are highly conserved among GRKs, and site-directed mutagenesis of these amino acids followed by kinetic analysis reveals pronounced defects in the phosphorylation of GPCR and soluble substrates (*i.e.* peptides or tubulin), regardless of the GRK subfamily (Huang et al., 2009; Pao et al., 2009; Sterne-Marr et al., 2009; Boguth et al., 2010; Huang et al., 2011). Thus, the inter-domain bridge formed by the N-terminal helix seems to be a critical structure for

maintaining GRKs in a catalytically competent state, consistent with previous studies indicating a critical role for the GRK N-terminal region in receptor phosphorylation (Palczewski et al., 1993). Indeed, introduction of a disulfide bond that covalently attaches GRK1 N-terminus to its C-tail improves catalytic efficiency (Huang et al., 2011).

The most recent GRK6 structure was not determined in complex with a GPCR. Thus, although it seems clear what needs to occur in the GRK to achieve an active conformation, it is less apparent how an activated receptor would stabilize this state, and which elements of the GRK comprise the receptor docking site. However, the new GRK6 structure provides some clues. Some of the residues in the N-terminal helix are highly conserved in all GRKs, yet face away from the kinase domain and are not involved in contacts with the small lobe or C-tail, interacting instead with their equivalents in a crystal contact to form an anti-parallel coiled-coil. The authors reasoned that this crystal contact could serve as a surrogate for an activated GPCR, thereby allowing GRK6 to assume a closed state. This model predicts that mutations of the residues involved should lead to defects in GPCR phosphorylation, but not peptide phosphorylation. This indeed turned out to be the case for GRK6 and GRK1 (Boguth et al., 2010; Huang et al., 2011), suggesting that these N-terminal residues form at least part of the GPCR docking site. There are thus interesting parallels between how heterotrimeric G proteins and GRKs are expected to interact with active GPCRs, as they both seem to use a protruding, amphipathic helix that is intrinsically disordered when not docked with a GPCR, as the primary receptor recognition motif (Huang and Tesmer, 2011). Other regions of the GRK undoubtedly also contribute to GPCR interactions, most likely in the C-tail and the small lobe. There have been reports of other regions outside the kinase domain that are suspected of making contributions to receptor binding (Dhami et al., 2004; Baameur et al., 2010), but these are less firmly established or could be receptor specific.

Most GRKs require the presence of negatively charged lipids to phosphorylate GPCRs efficiently, and they also require the cytoplasmic surface of the active receptor to form a pocket that is not accessible in the inactive state (Choe et al., 2011; Rasmussen et al., 2011; Standfuss et al., 2011) into which the GRK will dock. The size and physical properties of this pocket are expected to be highly conserved among receptors, while the size and sequence of the more exposed cytoplasmic loops of the receptor are clearly not. Structure of opsin in complex with a peptide derived from the C-terminus of $G\alpha_t$ demonstrates its potential to bind amphipathic helices (Scheerer et al., 2008), such as those now known to be contained in GRKs and heterotrimeric G proteins. The negative charge of the inner leaflet of the lipid bilayer would in turn be recognized by a complimentary positively charged surface of the GRK, such as that found immediately adjacent to the N-terminal helix of GRK6 (Boguth et al., 2010). Thus, a consistent model for GPCR-mediated GRK activation is one that involves the activated receptor in its native lipid environment forming a surface that is complimentary in shape and charge to that of GRKs in their active, closed conformation, which is approximated by the most recent structure of GRK6. Such a mechanism would allow GRKs to recognize a broad array of GPCR substrates. Once docked, the kinase adopts a closed conformation that allows it to phosphorylate any substrate in close proximity, although the docked receptor itself would be entropically favored. Effective phosphorylation of monomeric rhodopsin in nanodiscs by GRK1 has recently been demonstrated, which does not support the idea that GRKs dock to one receptor, and phosphorylate another associated with the first, as in a GPCR oligomer (Bayburt et al., 2011). The molecular details of how GRKs initially recognize receptors remains a matter of speculation, but one hypothesis is that their intrinsically disordered N-termini form an initial low-affinity interaction in what has been termed “fly-casting”, which is believed to kinetically favor complex formation (Shoemaker et al., 2000; Cortese et al., 2008).

5. GRKs phosphorylate non-GPCR substrates

An intriguing development in recent years has been a discovery of the ability of GRKs to interact with a variety of proteins other than GPCRs and in many cases to phosphorylate them (Table 1). The data extend the repertoire of pathways whose signaling is controlled by GRKs via phosphorylation of various signaling components. The list of non-GPCR substrates now includes single transmembrane domain tyrosine kinases (PDGFRβ), single transmembrane domain serine/threonine kinases, death receptors, toll-like receptors, transcription factors and adapter proteins. It remains unclear whether this mode of GRKs acting on non-GPCR-linked signaling pathways is an exception or a rule. If indeed GRKs participate not only in desensitization but also in signaling via such a huge variety of targets, they might play a role in processes such as cell growth and proliferation, cell death and motility, immunity, cancer, and development. As suggested above, one explanation is that only GPCR-bound active GRKs are able to phosphorylate proteins located in close proximity to these complexes. Two examples of this would be “high-gain” phosphorylation in the retina (Binder et al., 1990; Binder et al., 1996) and phosphorylation of IRS-1 by GRK2 in response to activation of endothelin receptors (Usui et al., 2005). Alternatively, GRKs might use their low intrinsic basal activity to phosphorylate non-GPCR substrates. It is also conceivable that at least some of the non-receptor substrates are able to activate GRKs in a manner similar to that of active GPCRs, albeit by distinct molecular mechanisms. This appears to be the case for tubulin (Pitcher et al., 1998; Carman et al., 1998) and synuclein, which are phosphorylated with higher catalytic efficiencies than peptide substrates (Pronin et al., 2000). GRK6A, but not its other splice variants, phosphorylates the Na^+/H^+ exchanger regulatory factor by forming an interaction between its C-terminus and the PDZ domain of the factor (Hall et al., 1999). Given the diversity of GRK non-receptor substrates that have already been described, the mechanism of GRK activation might be unique for each substrate.

GRKs can phosphorylate other classes of cell surface receptors. GRKs have been shown to phosphorylate the Smoothened (Smo), a seven transmembrane domain receptor belonging to a distant group within the GPCR superfamily. Smo is part of the evolutionary conserved hedgehog (Hh) signaling pathway. In the absence of Hh, Smo, the signal transducer component of the pathway, is inhibited by the Hh receptor Patched (Ptc). Hh binding to Ptc frees Smo to travel to the plasma membrane in *Drosophila* or to cilia in vertebrates, which it must do to signal. The main signaling pathway of Smo consists of several signaling events ultimately leading to the regulation of Ci (in *Drosophila*)/Gli (in vertebrates) family of transcription factors [reviewed in (Huangfu and Anderson, 2006)]. It remains controversial whether G proteins play any role in Smo signaling [reviewed in (Ayers and Thérond, 2010)], although recent data indicate that that in *Drosophila* Smo can function as canonical GPCR through coupling to Gi (Ogden et al., 2008). Recently, phosphorylation of Smo by GRKs has been shown to be a part of the Smo signal transduction cascade. A knockdown of Gprk2, a *Drosophila* ortholog of mammalian GRKs 4/5/6, diminishes Smo signaling and induces Hh loss-of-function (Zhang et al., 2007) phenotype (Molnar et al., 2007a; Cheng et al., 2010a). Gprk1, a *Drosophila* orthologous of mammalian GRKs 2/3, has also been implicated in mediating the Smo signaling, albeit less efficiently (Cheng et al., 2010a). Gprk2 activates Smo by phosphorylating it at $Ser^{741/742}$, and this phosphorylation is regulated by phosphorylation of adjacent sites by PKA and casein kinase (Chen et al., 2010b). Knockdown of Grk2/3 in zebrafish leads to defective Smo signaling and developmental abnormalities (Philipp et al., 2008), and GRK2-dependent phosphorylation of Smo has been shown to promote Smo signaling in mammalian cells (Meloni et al., 2006) suggesting a role of GRK2/3-mediated Smo phosphorylation in Smo signaling in vertebrates analogous to that in *Drosophila*. Interestingly, Gprk2-mediated Smo phosphorylation in *Drosophila* (Cheng et al., 2010a) and GRK2-dependent phosphorylation in mammalian cells

(Chen et al., 2004) induces arrestin recruitment, Smo internalization and down-regulation in a way reminiscent of "normal" GPCRs. However, if in canonical GPCRs, GRK/arrestin-dependent internalization and down-regulation is a part of the desensitization process, in *Drosophila* Gprk2-mediated Smo trafficking does not impair signaling (Cheng et al., 2010a). For many GPCRs, GRK- and arrestin-dependent internalization is the first step leading to degradation that occurs in continuing agonist presence. In contrast, Smo expression is low without the agonist, and Hh stabilizes Smo, whereas Gprk2 destabilizes it (Cheng et al., 2010a). Therefore, the way GRKs function in the Hh pathway is quite distinct from that in canonical GPCR signaling. Considering the importance of the Hh signaling pathway in developmental disorders and many forms of cancer [reviewed in (Jiang and Hui, 2008)], these findings implicating GRKs in the regulation of Smo signaling warrant further effort in elucidating the role of these kinases in the Hh pathway.

GRK2 and 5 phosphorylate platelet-derived growth factor receptor-β (PDGFRβ) (Freedman et al., 2002). The GRK2-dependent serine phosphorylation of PDGFRβ reduces Tyr autophosphorylation and allosteric activation of $G\alpha_i$ (Freedman et al., 2002), as well as the association of PDGFRβ with the Na^+/H^+ exchanger regulatory factor (Hildreth et al., 2004). In mouse aortic smooth muscle cells, it is GRK5 and not GRK2 that phosphorylates PDGFRβ, thus desensitizing PDGFRβ-induced phosphoinositide hydrolysis but enhancing Src activation (Wu et al., 2006). So far, however, PDGFRβ remains the only non-GPCR cell surface receptor shown to be desensitized in a GRK-dependent manner. EGF receptor is phosphorylated by GRK2 but no detectable functional consequences of this phosphorylation are documented (Freedman et al., 2002) (Hildreth et al., 2004).

GRKs have also been reported to phosphorylate a diverse collection of receptor-associated proteins, changing their binding to the receptor and/or their activity, which might lead to desensitization of G protein-dependent or independent signaling pathways. In most known cases, neither the mode of GRK activation nor the functional role of GRK-mediated phosphorylation is well understood. It is well established that activated GPCRs can enhance GRK-mediated phosphorylation of exogenous peptides up to 100-fold (Palczewski et al., 1991; Chen et al., 1993). Thus, GRKs should be able to phosphorylate other substrates under the same circumstances in cells. One example is arrestin-2. Serotonin 5-HT4 receptors activate cAMP-dependent signaling and MAP kinase signaling via G_s and Src, respectively, which associate with the receptor complex (Barthet et al., 2007). Upon 5-HT4 receptor stimulation, GRK5 phosphorylates a cluster of serine-threonine residues in the receptor C-tail and thereby promotes arrestin-2 binding. GRK5 then directly phosphorylates arrestin-2, which prevents activation of Src (Barthet et al., 2009). Although it is not a receptor that is phosphorylated, this clearly represents a variation of desensitization. GRK2-dependent phosphorylation of p38 might also be happening largely inside signaling complexes assembled on active receptors, because the association of GRK2 with p38 is agonist-dependent (Peregrin et al., 2006). There is at least one alternative mechanism that would make GRK-dependent phosphorylation of other proteins largely contingent on GPCR signaling. Receptor activation leads to the recruitment of GRKs and arrestins to GPCR-rich membranes (Daaka et al., 1997; Oakley et al., 2000), which greatly increases their local concentration, thereby making an encounter between a GRK and any receptor-associated or membrane-enriched substrate more likely.

GRKs are also able to phosphorylate and regulate nuclear proteins, such as class II histone deacetylases (HDACs) (Martini et al., 2008) and multiple transcription factors. In the case of HDAC, GRK5, which has a nuclear localization signal, is implicated (Johnson et al., 2004). GRK2, which lacks recognizable nuclear localization signal and is usually excluded from the nucleus in most cells was strongly implicated in the phosphorylation of transcription factors (Ho et al., 2005). In most known cases, phosphorylation occurs in response to

stimulation of specific membrane receptors, and thus it appears that transcription factors would need to be phosphorylated in the cytosol and travel to the nucleus afterwards. Indeed, GRK-mediated phosphorylation affects nuclear translocation of transcription factors as well as their activation (Ho et al., 2005; Patial et al., 2010). Phosphorylation of IκBα and nuclear translocation of NFκB p65 following stimulation of Toll-like receptor-4 by lipopolysaccharide were impaired in primary macrophages derived from GRK5 knockout mice, along with reduced inflammatory response to lipopolysaccharide (Patial et al., 2010). These data establish GRK5 as a positive regulator of the Toll-receptor-4-linked NFκB pathway. Conversely, GRK2 negatively regulates the transforming growth factor β (TNFβ)/ activin signaling pathway (Ho et al., 2005). Prolonged exposure to activin upregulates GRK2, which phosphorylates Smad2 and 3 preventing their phosphorylation by type I TNFβ receptor. The phosphorylation of these receptor-regulated Smads at carboxy-terminal serine residues by type I receptor kinase is necessary for their oligomerization and nuclear translocation (Moustakas and Heldin, 2009). As a result, GRK- dependent phosphorylation of Smads antagonizes TNFβ-mediated target gene expression and cellular effects (Ho et al., 2005). Purified GRK2 phosphorylates GST-Smad2 and 3, but in cells the formation of GRK2-Smad complex was activin-dependent (Ho et al., 2005). In other examples, GRK2 and 5 mediate tumor necrosis factor a α(TNFα)–induced activation of the NFkB transcription factor in mouse microphages via phosphorylation of IkBα (Patial et al., 2009). Conversely, GRK5-mediated phosphorylation of p105 stabilizes p105 and negatively regulates ERK1/2 activation via toll-like receptor 4 (Parameswaran et al., 2006).

Cytoskeletal targets of GRKs are also evident. Tubulin was the first non-GPCR substrate of GRKs described in 1998 (Haga et al., 1998; Pitcher et al., 1998; Carman et al., 1998). GRK2 and 5 phosphorylate tubulin, with the preference for β-tubulin and, under certain conditions, for the βIII-isotype (Carman et al., 1998). The finding that tubulin phosphorylation by GRK2 is enhanced by the addition of Gβγ and phospholipids (Haga et al., 1998; Carman et al., 1998), both of which promote GRK2 association with the plasma membrane, and by agonist-bound GPCRs (Haga et al., 1998; Pitcher et al., 1998) suggest once again that in cells GRK2 activated by GPCRs could be largely responsible for tubulin phosphorylation, which would localize this process to the vicinity of receptor-rich membranes. GRK2 also phosphorylates two other cytoskeletal proteins, ezrin and radixin, both members of ezrin-radixin-moesin (ERM) protein family (Fehon et al., 2010). GRK2 phosphorylates ezrin at a single Thr567 residue (Cant and Pitcher, 2005), which is important for maintaining ezrin in an active conformation with both the plasma membrane and F-actin binding domains accessible (Fehon et al., 2010). Similarly, GRK2 phosphorylates radixin at critical Thr564 residue (Fehon et al., 2010). GRK2 phosphorylation of ezrin is PIP_2- and Gβγ-dependent, suggesting that it is performed by the membrane-associated, possibly receptor-activated kinase. Furthermore, GRK2-mediated phosphorylation of ezrin is required for muscarinic M1 receptor-induced ruffle formation in Hep2 cells (Cant and Pitcher, 2005), again strongly suggesting the involvement of GPCR in the GRK2 activation. In contrast to ezrin, GRK2 phosphorylation of radixin was unaffected by PIP_2 and Gβα (Kahsai et al., 2009). Based on this limited data, it is reasonable to conclude that GRKs might transmit GPCR signaling to the cytoskeleton. Synucleins are another interesting class of proteins shown to be phosphorylated by GRKs. GRK2 and 5 phosphorylate synucleins very efficiently, with α- and β-synuclein being the best substrates for GRK2 and α-synuclein for GRK5 (Pronin et al., 2000). Lipids stimulated synuclein phosphorylation by both GRKs and lipids plus Gβγ strongly enhanced GRK2-dependent synuclein phosphorylation, which suggests that GPCRs might regulate synuclein phosphorylation by GRKs in cells. Although the function of synucleins remains unclear, they are of great medical interest because α-synuclein is the first gene to be implicated as a cause of a rare familial form of Parkinson's disease (Polymeropoulos et al., 1997).

6. Proteins regulated by GRKs in phosphorylation-independent manner

GRKs have been reported to regulate several signaling proteins via direct interaction that does not require kinase activity (Table 2). It is not unusual for enzymes to perform scaffolding functions in addition to or instead of their enzymatic activity. GRKs, particularly GRK2 and 3, are fairly large multidomain proteins, and it is conceivable that they could interact with a multitude of proteins via different domains (Pronin et al., 1997; Lodowski et al., 2003; Tesmer et al., 2005). As the number of proteins GRKs scaffold proliferate (Ribas et al., 2007) [as happened before with arrestins (Gurevich and Gurevich, 2006a)], it becomes particularly important to verify these interactions in physiological settings, elucidate how all these multiple interactions are organized in cells, whether GRKs scaffold proteins complexes in a receptor-dependent manner or are able to do so independently of receptor stimulation, and most importantly, to determine the biological roles of these interactions.

6.1. Receptor desensitization via the RH domain

Identification of a region within GRK N-terminus with a homology to RGS proteins (the RH domain) by *in silico* methods (Siderovski et al., 1996) led to discovery of a new way for GRK to suppress G protein-dependent signaling. Subsequent studies showed that the RH domain of GRK2 and 3 binds and sequesters active $G\alpha_q$ subunit (Table 3) (Carman et al., 1999; Usui et al., 2000; Tesmer et al., 2005). Canonical RGS proteins act as GTPase activating proteins (GAP) promoting GTPase activity of GTP-liganded α-subunit of a G protein and thus attenuating G protein-dependent signaling [reviewed in (Hollinger and Hepler, 2002)]. RH domains of GRKs, on the other hand, possess weak or no GAP activity, and their ability to attenuate signaling is primarily due to sequestering activated $G\alpha_{q/11}$ (Carman et al., 1999) or by direct blockade of the GPCR (Dhami et al., 2002). Another difference between RGS proteins and RH domains of GRKs is that most RGS proteins interact with $G\alpha_{i/o}$ as well as with $G\alpha_q$, with varying selectivity among RGS families (Ross and Wilkie, 2000; Hollinger and Hepler, 2002), whereas GRK RH is selective for $G\alpha_q$ (Carman et al., 1999; Sallese et al., 2000a) and is even able to discriminate among different members of $G\alpha_q$ family, binding $G\alpha_{11}$ and $G\alpha_{14}$, but not $G\alpha_{16}$ (Day et al., 2003). Although all GRKs possess RH domain, in members of the GRK1 or GRK4 subfamilies it does not seem to be able to bind any Gα (Carman et al., 1999; Picascia et al., 2004). Mutagenesis studies identified eight residues in the N-terminal region of GRK2 important for the $G\alpha_q$ binding, six of which are conserved in GRK3 but not in other members of GRK family (Sterne-Marr et al., 2003), which likely explains why only GRK2 and 3 bind $G\alpha_q$.

RH-mediated dampening of signaling via G_q-coupled GPCRs is an important regulatory mechanism, acting in concert with phosphorylation-dependent receptor desensitization in primary cells (Willets et al., 2004), or on endogenous receptor signaling in certain cultured cells (Luo et al., 2008). GRKs acting via RH can also interfere with GPCR-independent G_q-dependent processes. In one such case, insulin stimulates glucose transport acting via insulin receptor- dependent phosphorylation of $G\alpha_{q/11}$ (Imamura et al., 1999). GRK2 acting as a scavenger of $G\alpha_{q/11}$ inhibits insulin-induced glucose transport (Usui et al., 2004). Similarly, GRK2 mediates an inhibitory effect of chronic treatment with endothelin-1 on glucose transport by interfering with the $G\alpha_{q/11}$-dependent signaling in a phosphorylation-independent manner via its RH domain. Additionally, GRK2, in response to endothelin-1 treatment, phosphorylates insulin receptor substrate (IRS)-1 promoting its degradation and reducing insulin-dependent signaling (Usui et al., 2005). Therefore, both modes of GRK2 action contribute to endothelin-1-induced insulin resistance.

The GRK1 and GRK4 subfamilies also have structurally similar RH domains (Lodowski et al., 2003; Lodowski et al., 2006), which have not been reported to bind any G protein α-subunits (Carman et al., 1999; Day et al., 2004; Sterne-Marr et al., 2004). However, these

domains are found in all GRKs kinases, suggesting that they have biological functions that remain to be elucidated. One possibility is to modulate the conformation of the GRK kinase domain, as the RH domain bridges the kinase small and large lobes, thereby contributing to the low basal activity of the kinase (Lodowski et al., 2003). Indeed, in the relatively closed GRK6 structure the contacts between the RH domain and the large lobe are severed (Boguth et al., 2010). A second global function may be to fix the C-terminus of the C-tail of the kinase domain so that the so-called hydrophobic motif, common to all AGC kinases (Pearce et al., 2010), remains docked to the small lobe. As a result, an additional phosphorylation event is not needed to activate GRKs in response to the activation of GPCRs.

6.2. Regulation via other mechanisms

GRK2 and 3 also have the Gβγ binding site in their C-terminus (Koch et al., 1993; Touhara et al., 1994 ; Lodowski et al., 2003). It has long been considered a part of the mechanism responsible for transient recruitment of these kinases to the membrane upon GPCR activation (Haga and Haga, 1992; Pitcher et al., 1992; Li et al., 2003). Recently, a different function of the Gβγ-binding pleckstrin homology domain came to light, which is quite similar to the function of RH domain: desensitization of GPCR signaling by sequestering a G protein subunit, in this case Gβγ. G protein-coupled potassium channels (GIRK) are activated by *Pertussis* toxin-sensitive $G_{i/o}$-coupled GPCRs via Gβγ subunits [for review see (Sadjaa et al., 2003)]. GRK2, and likely GRK3, competitively binds Gβγ with high enough affinity to keep it away from GIRK, thereby reducing channel activation by GPCRs (Raveh et al., 2010). This is a novel mechanism of GPCR inactivation by GRK at the level of the effector, and it might be applicable to other Gβγ-dependent processes in cells (Raveh et al., 2010).

The discovery of GRK interacting proteins (GIT) GIT1 and GIT2 was the result of a search for proteins that might bind GRKs and serve as “effectors” (Premont et al., 1998) mediating GPCR signaling. GITs were the first proteins identified that bind GRKs without serving as substrates. GITs are multidomain scaffolding proteins that bind many partners, including ARFs, other small GTPases, and various kinases (Hoefen and Berk, 2006). The main function of GITs appears to be modulation of cytoskeletal dynamics during cell attachment and migration. It is significant that the first protein found to interact with GITs (after GRKs) was paxillin, an important component of focal adhesions (Turner et al., 1999). GITs bind another major component of focal adhesions, PIX, recruiting active Rac to its downstream effector PAK via PAK binding to PIX in GIT-PIX oligomers (Hoefen and Berk, 2006). Additional functions of GITs include their role in receptor internalization and membrane trafficking and scaffolding of signaling cascades (Hoefen and Berk, 2006). The ability of GRKs to interact with GITs might give GRKs a “say” in all these functions, including cytoskeletal remodeling. Recently it has been shown that GRK2 promotes integrin-dependent migration of epithelial cells towards fibronectin via interaction with GIT (Penela et al., 2008). The migration of epithelial cells was promoted by a lipid messenger sphingosine-1-phosphate (S1P) acting via Gi-coupled $S1P_{1/3}$ receptors, and the effect of GRK2 was dependent on the function of these receptors. The physiological importance of the GRK2-dependent coordination of integrin- and GPCR-directed cell migration is underscored by the delayed wound healing in Grk2 hemizygous mice (Penela et al., 2008). Interestingly, GRK2 acted as a positive modulator of the $S1P_{1/3}$ receptor-dependent signaling via the MEK/ERK pathway, suggesting that in this case it does act as an effector actually mediating rather than desensitizing the GPCR signaling. Importantly, GRK2 serves as an effector not in its kinase capacity, but as a scaffolding protein recruiting major players in regulating cell migration to the focal adhesion points.

GRKs have been shown to scaffold other signaling proteins with functional consequences. GRK2 negatively regulates ERK activation by chemokine receptor CCR2B via binding to

MEK or MEK-containing signaling complexes (Jiménez-Sainz et al., 2006; Luo et al., 2008). GRK2 phosphorylates CCR2B upon activation and desensitizes chemokine-induced calcium signaling (Aragay et al., 1998). However, the effect of GRK2 on ERK did not require kinase activity or G_q binding via the RH domain. Similar effects of GRK2 on chemokine-induced ERK activation were observed with heterologously expressed chemokine CXCR4 receptor and in splenocytes, which express endogenous CXCR4 receptor, from GRK2 hemizygous mice (Jiménez-Sainz et al., 2006). GRK2 can directly bind and inhibit another signaling molecule, a serine-threonine kinase Akt. GRK2 bound both active and inactive Akt, and the binding site was localized to the C-terminus. GRK2 binding to Akt reduced Akt activity and its ability to phosphorylate and activate nitric oxide synthase in sinusoidal endothelial liver cells (Liu et al., 2005b). Nitric oxide synthase is critical to maintain vascular homeostasis, and a defect in its activity seen in injured sinusoidal endothelial cells leads to intra-hepatic portal hypertension (Rockey and Chung, 1998; Shah et al., 1999; Yu et al., 2000). The expression of GRK2 in injured sinusoidal endothelial cells increased proportionally to the severity of the injury, and GRK2 knockdown increased phosphorylation of Akt, the Akt-mediated nitric oxide production, and ameliorated portal hypertension (Liu et al., 2005b). GRK2 was reported to directly interact with phosphoinosidite-3-kinase (PI3K) promoting its membrane localization, phosphoinositide production, AP-2 recruitment to the receptor, and receptor endocytosis (Nada Prasad et al., 2001; Naga Prasad et al., 2002). Thus, GRK2-mediated recruitment of PI3K to agonist-stimulated β-adrenoreceptors that facilitates receptor endocytosis is a contributing factor in the heart failure (Perrino et al., 2005a; Perrino et al., 2005b). PI3K is an upstream kinase in the Akt pathway (Manning and Cantley, 2007). Phosphoinositides produced by PI3K recruit phosphoinositide-dependent kinase 1 (PDK1) and Akt to the membrane, and PDK1 phosphorylates Akt at the regulatory residue Thr308 (Chan et al., 1999; Manning and Cantley, 2007). It would be of interest to determine whether the association of GRK2 with a PI3K signaling complex (Nada Prasad et al., 2001; Naga Prasad et al., 2002) affects PI3K activity and the downstream events such as Akt activity. GRK2-mediated recruitment of PI3K to the membrane should be expected to activate the Akt pathway, whereas direct GRK2-Akt interaction inhibits the Akt activity. It is unclear how this complex interplay of GRK2-mediated signaling events influences the control of physiological functions by the PI3K-Akt signaling pathway.

7. GRK regulation

The expression level, as well as activity of most enzymes in the cell is tightly regulated. GRKs are no exception. As described above, the best-known mechanism of GRK regulation is via direct binding to active GPCRs. However, this is just one of several established regulatory mechanisms.

7.1. Regulation of GRK activity

Like many kinases, GRKs are regulated by phosphorylation and protein-protein interactions (Table 4?). Gβγ subunits stimulate GRK2 and 3 via their PH domains 10–12-fold *in vitro* (Haga and Haga, 1992; Kim et al., 1993), likely due to enhanced recruitment of kinases to active receptors (Pitcher et al., 1992; Kim et al., 1993; Pitcher et al., 1995), although it is clear that Gβγ-binding also affects the conformation of the GRK2 kinase domain allosterically (Lodowski et al., 2005). GRK2 and 3 are also stimulated by anionic phospholipids such as phosphoinosidides (DebBurman et al., 1995; Onorato et al., 1995; Pitcher et al., 1995; DebBurman et al., 1996). In fact, the ability of Gβγ to stimulate GRK2 and recruit it to the membrane depends on the presence of anionic phospholipids (DebBurman et al., 1996; Pitcher et al., 1996). There is even a certain specificity of GRK interaction with Gβγ subunit: GRK2 binds Gβγ containing Gβ1 or Gβ2 but not Gβ3,

whereas GRK3 binds all three isoforms equally well (Daaka et al., 1997). In contrast, GRK5 is not activated by Gβγ subunits (Kunapuli and Benovic, 1993) but it is activated by binding to phospholipids such as phosphatidylinositol 4, 5-bisphosphate (PIP_2), as are other members of the subfamily, GRKs 4 and 6 (Pitcher et al., 1996). However, in contrast to the GRKs 2 and 3, which bind PIP_2 and other lipids via their PH domain, the members of the GRK4 subfamily seem to interact with PIP_2 via the N-terminus (Pitcher et al., 1995; Pitcher et al., 1996), presumably at the basic region close to the N-terminal helix in the GRK6 structure (Boguth et al., 2010).

GRK1 undergoes intramolecular autophosphorylation at Ser^{488} and Thr^{489} (Buczyłko et al., 1991; Palczewski et al., 1992). Autophosphorylation reduces the GRK1 activity towards phosphorylated light activated rhodopsin (Buczyłko et al., 1991; Pulvermüller et al., 1993; Palczewski et al., 1995) thereby serving as a negative feedback mechanism limiting the rate and extent of rhodopsin phosphorylation. GRK1 (at Ser^{21}) and GRK7 (at Ser^{23} and Ser^{36}) are phosphorylated by PKA in vitro and in cultured cells, which reduces their activity towards rhodopsin (Horner et al., 2005). GRK7 was found phosphorylated at the conserved PKA phosphorylation site Ser^{36} in dark-adapted cone photoreceptors in various species (Osawa et al., 2008). Using the *Xenopus laevis* retina as a model, it was shown that the level of the PKA-mediated phosphorylation of grk7 was diminished by light exposure (Osawa et al., 2008). Similarly, the level of GRK1 phosphorylation was higher in the dark-adapted mouse retina as compared to the light-adapted animals (Osawa et al., 2011). These data suggest that PKA phosphorylation of GRKs 1 and 7 serve as a feedback mechanism: dephosphorylation increases kinase activity upon light exposure when rapid opsin deactivation is required. Interestingly, phototransduction does not seem to be required for the light-induced dephosphorylation of GRK1, since it is preserved in mice lacking transducin α-subunit (Osawa et al., 2011).

GRK5 is regulated by a rapid phospholipid-stimulated autophosphorylation in its C-tail region at Ser^{484} and Thr^{485}, which enhances its ability to phosphorylate receptors (Kunapuli et al., 1994a). This is analogous to what happens at the so-called “turn motif” in other AGC kinases (Pearce et al., 2010). GRK5 is also phosphorylated in the C-terminus and inhibited by protein kinase C (PKC) (Pronin and Benovic, 1997). In contrast, GRK2 phosphorylated by PKC has higher activity towards rhodopsin (Chuang et al., 1995; Winstel et al., 1996). GRK2 is also phosphorylated by protein kinase A at Ser^{685}, which promotes its interaction with Gβγ and membrane recruitment (Cong et al., 2001). Similarly, GRK2 phosphorylation by cSrc, which is promoted by receptor stimulation, enhances the GRK2 activity towards receptors as well as non-receptor soluble substrates (Sarnago et al., 1999) and its ability to interact with $G\alpha_q$ (Mariggiò et al., 206). Thus, GRK2 phosphorylation by cSrc acts as a negative feedback augmenting receptor desensitization and reducing signaling. Conversely, agonist-induced GRK2 phosphorylation by ERK1/2 reduces the GRK2 activation by Gβγ and the ability to phosphorylate receptors (Pitcher et al., 1999; Elorza et al., 2000) functioning as a positive feedback loop enhancing G protein-mediated signaling. On the other hand, arrestin-mediated ERK activation, which is strongly enhanced by arrestin recruitment to active GPCRs (Luttrell et al., 2001), would be dampened. GRKs 2 and 5 are known to phosphorylate and desensitize a tyrosine kinase receptor PDGFRβ (Freeman et al., 2002; Hildreth et al., 2004; Wu et al., 2006). In turn, PDGFRβ phosphorylates and activates GRK2 in PDGF-dependent manner, which then phosphorylates and deactivates the receptor (Wu et al., 2005). A similar arrangement exists in case of GRK5 (Cai et al., 2009). The data on GRK regulation by phosphorylation appear to be incomplete: GRK2 and 5 are phosphorylated by multiple kinases, whereas no phosphorylation of GRK3, 4, and 6 is reported. In most studies, GRK2 and 5 served as representatives of their respective subfamilies, and there is no reason to expect that they are the only isoforms regulated via phosphorylation.

Calmodulin, caveolin-1, actin, and several less ubiquitous proteins were shown to affect GRK activity via direct binding. Mechanistically, the best understood case is inhibition of GRK1 and GRK7 by calcium-loaded recoverin, which directly competes with opsins for the kinase N-terminus (Chen et al., 1995a; Klenchin et al., 1995; Ames et al., 2006). An alternative mechanism of recoverin inhibition of GRK1 has been proposed involving a blockade by recoverin of a conformational change in GRK1 induced by active rhodopsin rather than direct competition for the binding site (Komolov et al., 2009). However, the concentration of Ca^{2+} in light-exposed rod outer segments seems insufficient to support complex formation, and recoverin actually translocates out of ROS in the light (Strissel et al., 2005), which casts doubts on the physiological relevance of this interaction. Although the phenotype of recoverin knockout mice is consistent with its role in Ca^{2+}-dependent inhibition of GRK1 (Makino et al., 2004; Sampath et al., 2005; Chen et al., 2010a), alternative explanations cannot be excluded. Inhibition of GRK7 by recoverin is much less studied than that of GRK1. A recent study has demonstrated that in the carp retina a non-mammalian recoverin homolog expressed in cones, visinin, provides a much broader range of Ca^{2+}-dependent regulation of the 7 activity than the range of the GRK1 regulation in rods (Arinobu et al., 2010) suggesting that recoverin x GRK7 interaction might be particularly functionally significant. Calmodulin binds to all GRK isoforms, but has a preference for GRK5 (Pronin et al., 1997). Calmodulin binding inhibits the GRK5 activity via reduction of its binding to lipids and stimulation of its autophosphorylation, which inhibits GRK5 interaction with the receptor. Calmodulin-dependent GRK5 autophosphorylation occurs at sites distinct from the two major residues Ser^{484} and Thr^{485} for phospholipid-stimulated autophosphorylation, which, in contrast to calmodulin-dependent autophosphorylation, activates the kinase (Kunapuli et al., 1994a; Premont et al., 1994). Calmodulin binds to a stretch of basic and hydrophobic residues in the N-terminal domain (Pronin et al., 1997), which is highly conserved in the subfamily suggesting that calmodulin might similarly regulate GRKs 4 and 6.

GRK2, in addition to being regulated by phosphorylation by multiple kinases, is S-nitrosylated at Cys^{340} by S-nitrosothiols and nitric oxide synthase following activation by multiple GPCRs (Whalen et al., 2007). S-nitrosylation inhibits the GRK2-mediated phosphorylation of βAR, recruitment of arrestins to the receptors, and receptor internalization and downregulation. This is a novel mode of regulation of the receptor desensitization, which may also be applicable to GRK3, since Cys^{340} is conserved in that isoform.

7.2. Regulation of GRK expression

The expression level of GRKs is known to be regulated by various factors and to be altered in pathological conditions. Chronic or even acute administration of GPCR agonists can increase the level of GRKs in the brain, which may lead to tolerance to drugs (Hurle, 2001; Diaz et al., 2002; Fan et al., 2002; Rubino et al., 2006; Schroeder et al., 2009). Administration of GPCR antagonists or removal of endogenous agonists also can affect the GRK concentration (Hurle, 2001; Diaz et al., 2002; Bezard et al., 2005; Ahmed et al., 2007; Ahmed et al., 2008; Ahmed et al., 2010). The concentration of GRKs *in vivo* and in cultured cells is responsive to various conditions such as stress (Taneja et al., 2011), neonatal ventral hippocampal lesion (Bychkov et al., 2010), cell cycle progression (Penela et al., 2010), and drug treatment (Salim et al., 2007). Human diseases alter GRK levels. The best-known case is the upregulation of GRK2 in the failing heart (Ungerer et al., 1993; Ungerer et al., 1994), but other pathological conditions also affect GRK expression (García-Sevilla et al., 1999; Grange-Midroit et al., 2003; Suo et al., 2004; Bychkov et al., 2008; Bychkov et al., 2011). In some cases, transcriptional regulation is involved, whereas in others alterations in the

protein concentration are not accompanied by changes in the mRNA levels, suggesting the regulation at posttranscriptional levels.

Little is known about the regulation of GRK transcription. Epinephrine acting simultaneously via α_2- and β2ARs upregulates the level of GRK3 mRNA and protein in neuronal cell lines (Salim et al., 2007). The action of epinephrine is mediated by the activation of ERK1/2 and possibly transcription factors Sp-1 and Ap-2. Interaction of GRK2 and 3 with the heat shock protein Hsp90 is critical for the maintenance of stable kinase levels in cells (Luo and Benovic, 2003; Salim and Eikenburg, 2007). GRK2 × Hsp90 interaction also seems to play a role in the proper folding and maturation of the newly synthesized GRK2. In the neuronal cell line BE(2)-C, interaction of GRK3 with Hsp90 aids in the kinase folding as well as increases its stability by suppressing proteosomal degradation (Salim and Eikenburg, 2007). The interaction with Hsp90 seems to be critical for folding and maturation of GRK5 and 6 as well (Luo and Benovic, 2003).

Relative abundance of mRNAs and proteins suggests that the half-lives of GRKs are widely different. GRK2 has the highest mRNA:protein ratio, suggesting that it is short-lived, followed by GRK3 (Gurevich et al., 2004; Bezard et al., 2005; Ahmed et al., 2007). Indeed, the half-life of GRK2 has been estimated at 6 min in HEK293, COS-7, Jurkat, and C glioma cells due to rapid degradation (Penela et al., 1998; Penela et al., 2001). Interestingly, in HL60 cells the GRK2 half- life was estimated at ~20 h and was reduced to 2 h by geldanamycin, an inhibitor of GRK2 interaction with Hsp90 (Luo and Benovic, 2003). Thus, the half-life of the same GRK isoform and presumably the mechanisms of its regulation in different cell types vary widely.

The regulation of GRK2 degradation is the most extensively studied, but little is known about other GRK subtypes. GRK2 is rapidly degraded via the proteasome pathway. Its degradation is facilitated by GPCR activation (Penela et al., 1998) and requires kinase activity (Penela et al., 2001). GRK2 is phosphorylated by cSrc *in vitro* and in cells (Sarnago et al., 1999). GRK2 phosphorylation by cSrc is facilitated by receptor activation (Sarnago et al., 1999) and promotes GRK2 degradation (Penela et al., 2001). cSrc phosphorylation sites have been localized to Tyr13, 86, and 92, and the Y13/86/92F GRK2 mutant is resistant to both cSrc-mediated phosphorylation and degradation (Penela et al., 2001). GRK2 is also phosphorylated by ERK (Pitcher et al., 1999; Elorza et al., 2000), and ERK-mediated phosphorylation of GRK2 not only reduces the GRK2 activity but also promotes its degradation (Elorza et al., 2003). Although cSrc- and ERK-mediated phosphorylation can target GRK2 for degradation independently, ERK preferentially phosphorylates GRK2 previously phosphorylated on tyrosine residues by cSrc (Elorza et al., 2003). GRK2 associates with and is ubiquitinated by Mdm2, which also facilitates GRK2 degradation (Salcedo et al., 2006). Mdm2-dependent GRK2 degradation upon GPCR activation is facilitated by previous GRK2 phosphorylation at Ser370 by MAP kinases but not by tyrosine phosphorylation (Nogués et al., 2011). Arrestins are known to recruit cSrc (Luttrell et al., 1999), ERK (Luttrell et al., 2001), and Mdm2 (Shenoy et al., 2001) to active GPCRs. Arrestin scaffolding activity is required for cSrc- (Penela et al., 2001), ERK- (Elorza et al., 2003), and Mdm2-mediated (Salcedo et al., 2006; Nogués et al., 2011) GRK2 degradation induced by GPCR activation. However, in the absence of GPCR activity, arrestins do not participate in the Mdm2-mediated regulation of the GRK2 basal turnover, but instead compete with GRK2 for Mdm2 and suppress basal GRK2 degradation (Nogués et al., 2011). Arrestin-dependent phosphorylation of GRK2 by cSrc, on the other hand, in addition to priming GRK2 for the phosphorylation by MAP kinase and subsequent Mdm2-dependent degradation upon GPCR activation (Sarnago et al., 1999), is able to drive Mdm2-independent basal GRK2 degradation in the absence of GPCR activity (Nogués et al., 2011). Thus, arrestins play a coordinating role recruiting kinases and/or ubiquitin ligases to GRK2

in the basal condition or upon activation of GPCRs, regulating GRK2 turnover via different pathways.

Dynamic regulation of the GRK2 expression during cell cycle progression depends on its phosphorylation at Ser^{370} by cyclin-dependent kinase 2 (CDK2) followed by the binding of a prolyl isomerase Pin1 to phosphorylated GRK2 (Penela et al., 2010). This sequence of evens is critical for the GRK2 degradation and its downregulation during G2 phase of the cell cycle. In contrast to other modes of regulation, CDK2-Pin1-dependent regulation of GRK2 degradation does not require arrestins (Penela et al., 2010).

The picture of the regulation of the GRK expression remains incomplete. The recent intriguing finding that females express more GRK3 and 5 in the brain than males, whereas the levels of GRK2 and 6 are essentially the same (Bychkov et al., 2010), indicates that sex-specific regulatory mechanisms might be involved.

8. GRK isoforms – more of the same?

No review of GRKs would be complete without discussion of receptor specificity of GRK isoforms. The situation with GRKs resembles that with arrestins (Gurevich and Gurevich, 2006a) in that there are too few of them to be strictly receptor specific. Thus, it is often assumed that GRK isoforms are “nonspecific” towards GPCRs. However, the issue is not as simple as it appears. There are just too many GRK isoforms preserved over millions of years of vertebrate and mammalian evolution to believe that they are promiscuous and entirely interchangeable. Even if we ignore highly specialized visual isoforms (GRKs 1 and 7), there are still two subfamilies, GRK2/3 and GRK4, comprising two and three members, respectively, not counting splice variants, so there must be a functional reason for their existence.

There are several aspects to the issue of receptor specificity. First question is whether any GRK is capable of phosphorylating any receptor. Such question is answered by testing receptor phosphorylation *in vitro* with purified GRKs and purified receptors in natural or artificial membranes. Due to easy availability, by far the most popular preparation has been purified rhodopsin in disk membranes as a substrate. The answer to the initial question seems to be “yes”, as all GRKs are biochemically capable of phosphorylating any receptor, although not necessarily with the same efficacy. Rhodopsin is a non-cognate receptor for all GRKs except GRK1, and yet all GRK isoforms phosphorylate it, indicating some degree of biochemical promiscuity. GRK6 is an isoform that acts as very weak kinase for rhodopsin (5% activity of GRK2 (Benovic and Gomez, 1993)). GRK6 phosphorylates β2AR somewhat better (35% of the GRK2 activity; (Benovic and Gomez, 1993)). GRK5, a member of the same GRK4/5/6 subfamily, is significantly less active towards rhodopsin, β2AR, and muscarinic M2 receptor than GRK2, but it is a much stronger kinase for rhodopsin and a slightly better kinase for β2AR than GRK6 (Kunapuli et al., 1994b). The deletion of the C-terminal sequence in GRK6C splice variant results in a significantly higher kinase activity towards light-activated rhodopsin than that of the longer variant GRK6A (which was originally cloned and most extensively studied) and the longest GRK6B (Vatter et al., 2005). It remains to be seen whether GRK6 has generally lower kinase activity than other isoforms or simply stricter receptor requirements that are still undiscovered. Some receptor preferences evident in the *in vitro* studies with purified proteins are common for GRK subfamilies, such as the ability of GRK2 and 3 to phosphorylate muscarinic M3 receptor, which is not significantly phosphorylated by GRKs 5 or 6 (Debburman et al., 1995). The repertoire of GPCRs available in purified form for *in vitro* phosphorylation studies has been so far quite limited. A wider array of receptors might yet reveal unexpected biochemical receptor specificity of GRK isoforms.

Second question is how different GRKs operate in living cells. Each cell expresses many GPCRs and more than one GRK isoform. In and of itself, it does not mean that any GRK has a chance to interact with any receptor. A cell is a highly compartmentalized environment and, consequently, some GRK isoforms and GPCRs subtypes may never meet. Furthermore, there might be conditions in a particular cell type, such as the presence of GRK modulating proteins, discouraging or precluding GRK interaction with particular GPCRs. Another consideration is the cellular complement of GRK isoforms and relative expression levels. Just because a GRK can phosphorylate a receptor does not mean it would ever have a chance, simply because another more abundant isoform would always get there first. These finer points are hard to appreciate in experiments involving heterologously expressed, and overexpressed, receptors and kinases. Receptors or GRKs may not localize to proper cellular compartments due to unnaturally high level of expression and, if they are not native to the cells they are expressed in, may not be subject to proper regulatory influences. An overexpressed kinase due to its abundance would always have an edge over endogenous proteins. Recent studies of endogenously expressed receptors and kinases proved that GRK isoforms could be quite selective towards receptors. These studies employed knockdown of endogenous GRKs or used dominant-negative constructs to interfere with the kinase functions. HEK293 cells have a full complement of non-visual GRK isoforms. Of these, GRKs 2, 3, and 6 apparently participate in desensitization of endogenous muscarinic M3 receptors, since knockdown of these isoforms enhanced carbachol-induced calcium mobilization, whereas GRK5 knockdown was ineffective (Luo et al., 2008). In HEK293 cells, GRK2 and 6 were chiefly responsible for arrestin recruitment to the heterologously expressed β2 adrenergic receptor (Violin et al., 2006). In contrast, in U2-OS osteosarcoma cells, which express little or no GRK6, GRKs 2 and 3 were the most efficacious. These data stress the contribution of the cellular complement of GRKs to the apparent “receptor specificity”. In human neuroblastoma SH-SY5Y cells, GRK6, but not GRKs 2, 3, or 5, phosphorylated and desensitized endogenous M3 muscarinic receptor (Willets et al., 2002; Willets et al., 2003). Interestingly, in the *in vitro* experiments, neither GRK nor GRK phosphorylated purified M3 receptor, whereas both GRK2 and 3 phosphorylated it (Debburman et al., 1995). One has to draw inevitable, although discouraging, conclusion that the receptor preference found *in vitro* may not be the same a GRK would exhibit in cells. In adult cultured cardiac myocytes, GRK3 effectively desensitized endogenous endothelin-1 and α_1-adrenergic receptors, whereas GRK2 was much less effective (Vinge et al., 2007). Conversely, both kinases desensitized the β_1 adrenergic receptor with similar efficacy, although the efficacy of GRK3 at the β_1 receptor was 20-fold lower than at the endothelin-1 receptor. GRK3 also seems responsible for desensitization of α_1-adrenergic receptors in the heart of living mice (Eckhart et al., 2000; Vinge et al., 2008), whereas GRKs 2 and 5 regulate β-adrenergic receptor-mediated responses (Koch et al., 1995; Rockman et al., 1996; Iaccarino et al., 1998). These are interesting observations demonstrating that even such closely related kinases as GRK2 and 3 may act quite differently in their native milieu. One possible mechanism could be the differential preference of GRK2 and 3 not for the receptors but for Gβγ isoforms necessary to recruit these kinases to active GPCRs (Daaka et al., 1997). In that study, GRK2 was found to form a complex with Gβγ following stimulation of endogenous β2AR and lysophosphatidic acid receptors but not thrombin receptors, whereas GRK3 interacted with Gβγ after stimulation of all three GPCRs (Daaka et al., 1997). If different receptors engage different Gβγ isoforms, then a preference of a GRK for particular Gβγ would result in some receptors not recruiting it and, therefore, not being regulated by it.

Studies with GRK knockout mice contributed their share of complications to the question of receptor specificity of GRKs. For supposedly promiscuous kinases, knockout of individual GRK isoforms resulted in mild, yet surprisingly distinct phenotypes. Apparently, mammalian GRKs have partially overlapping receptor specificity, so that remaining GRK

isoforms successfully compensate for the missing one in most, but not all, cases. The only exception was GRK2, knockout of which in mice is embryonic lethal due to abnormal formation of the heart (Jaber et al., 1996;). Apparently, this lethality stems from general, albeit undefined, role of GRK2 in embryogenesis, rather than specific role in the heart development, since mice with GRK2 ablation specific to the cardiac myocytes develop normally (Matkovich et al., 2006). Other GRK knockout lines display deficits in specific functions. Knockout of GRK3, the closest relative of GRK2, resulted in complete loss of olfactory function (Peppel et al., 1997), which is likely explained not by particular specificity of GRK3 for odorant receptors but by the fact that GRK3 is the major, perhaps the only GRK expressed in the olfactory epithelium (Peppel et al., 1997; Gurevich et al., 2004). Mice lacking GRK3 are resistant to κ opioid-induced dysphoria (Bruchas et al., 2007) and show reduced tolerance to opioid analgesics (McLaughlin et al., 2004; Terman et al., 2004). The mice with GRK5 deletion are functionally supersensitive to muscarinic, but not dopaminergic, stimulation (Gainetdinov et al., 1999) and have reduced hippocampal acetylcholine release due to impaired desensitization of M2/M4 autoreceptors (Liu et al., 2009). Conversely, mice lacking GRK6 show supersensitive behavioral responses to dopaminergic stimulation (Gainetdinov et al., 2003) and to certain effects of morphine (Raehal et al., 2009). Obviously, not all defects in GRK knockout have been documented yet, but there is enough evidence to conclude that GRK isoforms are not entirely interchangeable, and i*n vivo* some have very defined preference for certain GPCRs. This preference might not necessarily stem from any biochemical specificity of GRK isoforms, but rather from cell specific expression, differential subcellular targeting, and different functional consequences of receptor phosphorylation by different GRKs.

There is another important aspect of the “receptor specificity” question. Do different GRK isoforms phosphorylate receptors at the same sites? Does receptor phosphorylation by different isoforms lead to the same functional consequences? The answer to both questions is very likely “no”. In HEK293 cells, GRK2 and 3 mediated most of the angiotensin II-induced phosphorylation of angiotensin 1A receptor, arrestin recruitment, and receptor endocytosis, whereas GRK5 and 6 facilitated arrestin-mediated ERK activation (Kim et al., 2005). A similar picture was found for the V2 vasopressin receptor (Ren et al., 2005). GRK2, 3, and 6, but not GRK5, desensitized carbachol-induced calcium mobilization via endogenous M3 muscarinic receptor to a similar degree, with GRK2 (and possibly GRK3) acting via $G\alpha_q$ scavenging, and GRK6 via phosphorylation (Luo et al., 2008). At the same time, only GRK2 significantly participated in desensitization of the carbachol-induced ERK1/2 activation. Multiple endogenous ligands of the same receptors may engage different GRKs. Thus, stimulation of the CCR chemokine receptor with its endogenous agonist CCL19 leads to receptor phosphorylation by GRK3 and 6, whereas another endogenous agonist CCL21 recruits only GRK6 (Zidar et al., 2009). As a result, only CCL19 caused receptor desensitization, whereas both ligands induced arrestin-dependent ERK activation (Kohout et al., 2004; Zidar et al., 2009). These data strongly suggest that receptor phosphorylation by different GRKs differentially regulates subsequent signaling.

In general, the issue of receptor specificity of different GRK isoforms remains wide open. More biochemical experiments with much wider range of purified receptors must be performed. However, we also need to keep in mind obvious limitations of this approach: these experiments show how well a particular kinase can phosphorylate certain GPCR subtype, but cannot prove that it actually performs this function *in vivo*. The best example of this is the demonstration right after the discovery of GRK2 that it specifically phosphorylates light-activated rhodopsin (Benovic et al., 1986b), along with equally convincing demonstration that *in vivo* knockout of GRK1 in animals expressing normal complement of all other GRKs completely abolishes rhodopsin phosphorylation in rods (Chen et al., 1999a). Emerging evidence indicates that biologically relevant receptor

specificity of each GRK isoform and splice variant is largely determined by its co-expression with particular GPCRs in specific cell types and co-localization to the same sub-cellular compartments, along with additional regulatory mechanisms that enable or suppress its activity in the right place at the right time. A lot of additional *in vivo* experimentation using cell type-specific knockouts and knockdowns is necessary to determine which GRK(s) regulate each aspect of the function of each GPCR in physiologically relevant situations in living animals.

9. Physiological and pathological roles of GRK isoforms

In spite of the obvious importance of GRKs for the regulation of the GPCR signaling, their physiological functions remain poorly understood. Although the vertebrate GRK family is not large – only 7 members – it is functionally quite diverse, with two members, GRKs 1 and 7, playing specialized roles in photoreceptor cells and others ubiquitously expressed throughout the body obviously fulfilling multiple needs. To sort out all these complex interlinked functions played by individual GRK isoforms in different cell types has been, is, and will be an enormous challenge.

9. 1. The “visual” branch of the GRK family

The vertebrate retina contains two types of photoreceptor cells, rods and cones. Cones are active in bright light (photopic vision) and responsible for color vision. Rods, due to their high sensitivity and low noise, are adapted to night (scotopic) vision and rapidly become desensitized in bright light. Vertebrate GRKs 1 and 7 are also known as rhodopsin and cone opsin kinase, respectively. GRK7 is expressed in cone photoreceptor cells, whereas GRK1 is expressed in rods or in both rods and cones, depending on the species (Pugh and Lamb, 2000; Weiss et al., 2001; Wada et al., 2006). GRK1 is also expressed in the pineal gland (Somers and Klein, 1984; Blackshaw and Snyder, 1997; Zhao et al., 1997; Zhao et al., 1999) that has, for reasons unknown, a full complement of “visual” proteins (Blackshaw and Snyder, 1997). GRK1 was the first GRK identified, and its function is well defined and relatively well understood. GRK1 phosphorylates rhodopsin setting the stage for the high affinity binding of arrestin-1 (a.k.a. rod arrestin or visual arrestin) and the shut-off of the photoreceptor response to light. In mice expressing truncated rhodopsin with GRK1 phosphorylation sites eliminated (Chen et al., 1995b) or lacking the GRK1 gene (Chen et al., 1999a) the responses to light were abnormally prolonged, which proved the critical role of GRK1 in rhodopsin desensitization. Furthermore, rod photoreceptors in mice lacking GRK1 had shorter outer segments and, if exposed to constant dim light, underwent apoptosis and died (Chen et al., 1999a). Apparently, rod degeneration is associated with abnormally prolonged activation of the signaling cascade, since similar outer segment shortening and degeneration is observed in mice lacking arrestin-1 (Xu et al., 1997), as both outer segment damage and photoreceptor loss could be reversed by dark rearing or expression in GRK1 null mice of mutant arrestin-1 able to bypass GRK1 and to bind unphosphorylated rhodopsin (Song et al., 2009). In humans, mutations compromising the catalytic ability of GRK1 lead to Oguchi disease, a rare hereditary form of stationary night blindness (Yamamoto et al., 1997; Khani et al., 1998; Zhang et al., 2005; Hayashi et al., 2007; Oishi et al., 2007) characterized by prolonged insensitivity to low light after light exposure but generally normal acuity of vision in bright light. In contrast to mice, in human patients loss of GRK1 activity does not lead to photoreceptor degeneration, but results in night blindness and reduced light sensitivity of cones (Cideciyan et al., 1998). A light-independent retinal degeneration in GRK1 knockout mice has also been reported suggesting a previously appreciated role of GRK1 in rod photoreceptor survival (Fan et al., 2010).

Hyper-phosphorylation of rhodopsin by GRK1 could also be deadly for photoreceptors. In humans, multiple mutations in rhodopsin cause a group of retinal degenerative diseases

known as retinitis pigmentosa (RP), which are characterized by variable loss of rod photoreceptors across the retina followed by the death of cone photoreceptors (Mendes et al., 2005). RP is first experienced as night blindness, then loss of peripheral vision, and, ultimately, loss of central vision. Some of the mutations cause autosomal recessive RP and congenital stationary night blindness but most are autosomal dominant. Several mutations of Arg^{135} (R135K, R135Q, R135A, R135L, R135W) result in increased phosphorylation of the mutant opsin by GRK1, even in the absence of opsin ligand 11-*cis*-retinal, and enhanced arrestin binding (Shi et al., 2005). Importantly, these mutations cause a very aggressive form of PR with early age of onset and rapid progression (Andréasson et al., 1992; Ponjavic et al., 1997; Oh et al., 2004; Shi et al., 2005). Although the exact mechanisms of photoreceptor death caused by hyper-phosphorylation of the mutant opsins and enhanced arrestin binding remains unknown, it is possible that tight rhodopsin-arrestin association might cause rhodopsin to mislocalize in photoreceptor cells causing their demise (Chuang et al., 2004). Another mutation in rhodopsin implicated in autosomal dominant RP, K296E, results is constitutively active rhodopsin (Robinson et al., 1992), suggesting that persistent activation of the downstream signaling pathway might be the cause of photoreceptor degeneration. However, in mouse photoreceptors there was no light-independent activation, since the mutant rhodopsin was hyper-phosphorylated and bound to arrestin (Li et al., 1995). Apparently, both constitutive activation of transducin and stable complex with arrestin can contribute to photoreceptor death, since retinal degeneration is transgenic mice expressing mutant rhodopsin was ameliorated by the deletion of both transducin and arrestin, but not by either protein alone (Chen et al., 2006). Obviously, since human RP patients with the K296E mutation retain full complement of arrestin, the formation of the stable complex of phosphorylated rhodopsin with arrestin is likely the leading cause of photoreceptor death. Similarly, mice overexpressing GRK1 show photoreceptor damage when exposed to bright light (Whitcomb et al., 2010), suggesting that enhanced rhodopsin phosphorylation by GRK1 might compromise rod viability. Interestingly, the mechanisms of photoreceptor death caused by mislocalization of rhodopsin via its too tight binding to arrestin is evolutionarily conserved, since similar phenomenon is observed in *Drosophila* (Alloway et al., 2000; Kiselev et al., 2000)

Because GRK1 is expressed in cones in many species, it might participate in opsin phosphorylation and regulation of cone responses. In species that have in cones both GRKs 1 and 7, including humans, the defects in GRK1 do not completely compromise the function of cones (for example, in Oguchi disease), since they have functional GRK7 to phosphorylate opsins (Chen et al., 2001a; Liu et al., 2005a). Cones are designed to function in bright light and have to make an extra effort to recover from the bright light assault. For that purpose, evolution equipped cones with more elaborate and efficient machinery to shut off the light signal than that found in rods [for brief review see (Gurevich and Gurevich, 2010b)]. GRK7 is a part of this efficient shutoff machinery to allow for quick recovery. GRK7 is expressed at a very high level in cones [10 times more abundant than GRK1 in the carp retina (Tachibanaki et al., 2005)] and it has ~10 times higher specific activity than GRK1 leading to a rapid phosphorylation of cone opsins (Tachibanaki et al., 2001; Tachibanaki et al., 2005; Wada et al., 2006). GRK7 is geranylgeranylated (Hisatomi et al., 1998), which ensures its constant presence at the disk membrane in close proximity to cone opsins contributing to the highly efficient photoresponse shutdown. Knockdown of Grk7 in zebrafish with morpholino antisense nucleotides resulted in severely delayed photoresponse recovery and reduced temporal contrast sensitivity under bright light conditions (Rinner et al., 2005), indicating that GRK7 plays an important role in light adaptation in cones. Interestingly, one study has demonstrated normal deactivation kinetics in human long/medium wavelength cones in the absence of GRK7, which contrasted sharply with slower kinetics resulting from the loss of the GRK1 function in Oguchi disease (Cideciyan et al., 2003). These data argue against the predominant position of GRK7 in the light adaptation in

human cone photoreceptors and suggest that GRK1 plays the leading role in light adaptation in both rods and cones. The issue is far from resolved, and one interesting approach would be to compare the behavior of the human cones with that of the cones express only GRK7 [e.g., in pigs and dogs (Weiss et al., 2001)].

Interestingly, mice and rats do not express GRK7 in cones, and GRK1 is expressed in both rods and cones (Lyubarsky et al., 2000; Weiss et al., 2001). There is a report that GRK7 in the mouse is expressed in many tissues including the brain, olfactory bulb, lung, pancreas, and in the retina in the inner nuclear layer, but not in the photoreceptor layer (Chen et al., 2001a), but the absence of GRK7 gene in mouse genome (Caenepeel et al., 2004) suggests that presumably GRK7-specific antibodies used in this study cross-reacted with some other GRK isoform. In mouse cones, cone opsins are phosphorylated by GRK1 in the light-dependent manner, and photopigment phosphorylation is followed by the binding of cone-specific arrestin-4 to phosphorylated opsins (Zhu et al., 2003). Critical role of GRK1 in cone light adaptation in rodents is supported by the evidence that deletion of GRK1 in mice leads to profoundly slowed recovery of cone photoresponses (Lyubarsky et al., 2000).

Strictly speaking, invertebrates do not have the “visual” branch of the GRK family, that is, they do not have orthologs of GRKs 1 and 7. Therefore, invertebrate photoresponses are regulated by other GRK isoforms. In *Drosophila*, Gprk1, the ortholog of the mammalian GRK2/3, is enriched in photoreceptors and regulates the amplitude of visual responses (Lee et al., 2004), although it is also expressed in other tissues (Cassill et al., 1991). In octopus, a GRK termed rhodopsin kinase but highly homologous to the mammalian GRKs 2 and 3 is expressed exclusively in the retina and capable of phosphorylating rhodopsin (Kikkawa et al., 1998).

9.2. GRKs in the affaires of the heart

Heart function is regulated by βARs that are under control of circulating sympathetic catecholamines. Any kind of load or stress on the heart leads to the release of epinephrine from the adrenal glands and norepinephrine from sympathetic neurons. These hormones activate βAR expressed by cardiomyocytes. Active βARs activate Gs, which in turn increases adenylyl cyclase activity. Elevation of cAMP initiates a signaling cascade resulting in an acute adaptive increase in the heart output, which serves as a negative feedback loop suppressing further sympathetic activity. At the same time, receptor activation initiates GRK-dependent desensitization process leading to the signal shutoff. Under normal circumstances, this system acutely adjusts the cardiac output to the demands of the moment. Clinical data (Galetta et al., 2010) and experimental studies with mice overexpressing βAR (Engelhardt et al., 1999; Liggett et al., 2000) or Gs (Iwase et al., 1997) in the heart indicated that persistent βAR activity is detrimental to the heart, suggesting that GRK-mediated functional uncoupling of βAR is protective. This notion received support from a recent study in mice with cardiac-specific ablation of GRK2 that rapidly developed cardiomyopathy when treated continuously with β-adrenergic agonist isoproterenol (Matkovich et al., 2006). In the failing heart, a reduction in the cardiac output initiates a vicious cycle of unremitting sympathetic activation, continuous stimulation of βAR eventually resulting in their profound desensitization and loss of responsiveness to catecholamines over time [for a brief review see (Eschenhagen, 2008)]. Almost two decades ago, it has been found that marked desensitization of βAR in the failing heart is accompanied by upregulation of GRK2 at the protein and mRNA levels (Ungerer et al., 1993; Ungerer et al., 1994). It was reasonable to assume that the increase in the GRK2 concentration exacerbated the unresponsiveness of βAR by facilitating its phosphorylation and desensitization (Fig. 2). Indeed, overexpression of GRK2 in the heart resulting in attenuation of isoproterenol- stimulated contractility and reduced cAMP production (Koch et al., 1995), impairs cardiac function (Chen et al., 1998) and promotes apoptosis (Brinks et al., 2010) following ischemia and reperfusion injury.

GRK2 is not the only GRK isoform expressed in the heart. Overexpression of GRK3, which is closely related to GRK2 biochemically and is co-expressed in cardiomyocytes, did not alter the *in vivo* cardiac responsiveness to β-adrenergic stimulation (Iaccarino et al., 1998). Inhibition of GRK3 in the heart of transgenic mice via cardiac-specific expression of a dominant-negative construct analogous to that used for GRK2 (see below) increased sensitivity to stimulation of α1-adrenoreceptors and caused elevated systolic blood pressure due to increased cardiac output (Vinge et al., 2008). Cardiac-specific overexpression of the second major GRK isoform, GRK5, diminished cardiac responsiveness to isoproterenol (Rockman et al., 1996) and compromised cardiac functions (Chen et al., 2001b), suggesting that overactive GRK5 also might play a role in dysregulation of β-adrenergic signaling in heart failure. Furthermore, upregulation of GRK5 protein and mRNA has been reported in experimental models of heart failure (Ping et al., 1997; Takagi et al., 1999), although no evidence is yet available that such upregulation occurs in the human heart failure. A recent study painted a different picture of the GRK5 role in the heart functions. There is a L41Q polymorphism in the coding region of the GRK5 gene. L41 yields the enzyme with higher activity that Q41 (Liggett et al., 2008). The L41 polymorphism, common in African Americans, is protective against experimental cardiomyopathy, and is associated in human patients with lower mortality from heart failure (Liggett et al., 2008). It is important to note that mouse studies were performed under conditions of massive overexpression (~30 fold), which might have influenced the results. However, there is a report that L41Q polymorphism is associated with transient left ventricular apical ballooning syndrome, a stress-induced acute cardiac dysfunction, in Caucasian patients (Spinelli et al., 2010), suggesting that increased GRK5 activity can be harmful for the cardiac function.

If elevated level and resulting exaggerated GRK2 activity is a significant contributor to heart failure, then inhibiting or inactivating the kinase should be the goal (Fig. 2). Numerous attempts utilizing various strategies have been made to prove the point by suppressing the function of GRK2 with the aim of preventing heart failure. Transgenic or virus-mediated expression in the heart of the C-terminal GRK2 peptide βARKct that had a dominant-negative effect by competing with endogenous GRK2 for the binding to Gβγ and membrane recruitment has been widely used. Overexpression of βARKct was shown to be generally successful in preventing cardiomyopathy (Rockman et al., 1998b; White et al., 2000; Freeman et al., 2001; Harding et al., 2001; Shah et al., 2001; Rengo et al., 2009), as well as in reversing exaggerated desensitization of βAR (Rockman et al., 1998b; White et al., 2000; Shah et al., 2001; Eckhart and Koch, 2002) and constraining ischemic injury (Brinks et al., 2010) in animal models of heart failure and in explanted human heart (Williams et al., 2004). Mice hemizygous for GRK2 [homozygous knockout is embryonic lethal (Jaber et al., 1996;)] have increased myocardial contractility in response to isoproterenol stimulation, which is further improved in GRK2 +/− mice expressing βARKct (Rockman et al., 1998a). A caveat of using βARKct as a tool to manipulate the GRK2 function is that there are doubts as to its mechanism of action *in vivo*. βARKct is presumed to compete with wild type GRK2 for binding to Gβγ, thus impeding GRK2 recruitment to active receptors and, consequently, reducing receptor phosphorylation. However, a recent report suggested an alternative mechanism of the βARKct action (Völkers et al., 2011). βARKct virally delivered to cultured adult rat cardiomyocytes enhanced the cardiac L-type Ca^{2+} channel current following βAR stimulation by sequestering Gβγ and preventing its inhibitory interaction with the channel. As a result, βARKct improved contractile responsiveness to βAR without interfering with βAR-evoked cAMP signaling. This means that βARKct completely bypassed GRK2 as a target. Conditional ablation of GRK2 allowed for examination of the effect of GRK2 absence on the cardiac adaptation not only before but also after the myocardial infarction. Mice with GRK2 deleted before the infarction demonstrated improvement in post-infarction cardiac performance and βAR function similar to that seen

with the βARKct overexpression. Surprisingly, deletion of GRK2 even 10 days after the infarction also improved survival and cardiac contractile performance (Raake et al., 2008).

An alternative rather obvious target for therapy in heart failure is an elevated catecholamine level due to the heightened activity of the sympathetic nervous system, which is a conspicuous feature of this pathological condition. Ironically, GRK2 turned out to be the culprit in that, too. The sympathetic nervous system function is negatively regulated via α2-adrenoreceptors that act as autoreceptors, suppressing release of catecholamines (Brede et al., 2003). Heart failure in mouse and rat models was accompanied by adrenal hypertrophy, downregulation of adrenal α2-adrenoreceptors, and upregulation of GRK2 (Lymperopoulos et al., 2007). Attenuation of GRK2 activity by expression of its inhibitor βARKct restored α2-adrenoreceptor-mediated inhibition of the catecholamine secretion and improved the function of the failing heart. Recent data proved that beneficial effect of the exercise training, which is known to reduce morbidity and mortality in chronic heart failure, might stem from the reduction in the adrenal expression of GRK2 (Rengo et al., 2010). This reduction leads to restoration of the inhibitory influence of α2-adrenoreceptors on the sympathetic system, thus breaking the pathological cycle of enhanced catecholamine release and persistent adrenergic stimulation of the heart. As a result, the GRK2 level in the heart and βAR-stimulated cardiac contractility were also normalized (Rengo et al., 2010).

βARs are critical regulators of the function of the cardiovascular system. In cardiomyocytes, βARs regulate the heart activity, whereas βARs expressed in the smooth muscle of the blood vessels regulate the vascular tone. Impairment in βAR-mediated vasodilation has been implicated in hypertension in both human patients and animal models (Feldman, 1990; Feldman and Gros, 1998; Izzo et al., 2008). Similarly to heart failure, deficits in βAR-mediated signaling is accompanied by an upregulation of GRK2 in the vasculature of hypertensive people and animals (Gros et al., 2000; Cohn et al., 2008; Cohn et al., 2009). The GRK2 activity and expression also increases in circulating lymphocytes of hypertensive human patients (Gros et al., 1997; Gros et al., 1999) and animals (Gros et al., 2000) leading to reduced βAR-mediated stimulation of adenylyl cyclase activity (Gros et al., 1999). The level of GRK2 in lymphocytes may serve as a useful marker to monitor heart failure, since the expression of GRK2 in lymphocytes mirrors the myocardial expression of GRK2 (Hata et al., 2006; Oyama et al., 2006; Bonita et al., 2010).

Targeted vesicular smooth muscle overexpression of GRK2 leads to attenuated vasodilation in response to β-adrenergic agonist isoproterenol and elevation of conscious mean blood pressure (Eckhart et al., 2002). Disappointingly, the “tried-and-true” method of inhibiting the GRK2 activity in the vesicular smooth muscle with βARKct or knockdown failed to bring down blood pressure in the two-kidney, one-clip model of hypertension despite increased βAR-mediated vasodilation. The culprit was increased α1D-adrenoreceptor-mediated vasoconstriction that was also facilitated by GRK2 inhibition (Cohn et al., 2008). As in the heart, the second major GRK isoform expressed in vesicular smooth muscle is GRK5. Targeted overexpression of GRK5 increased blood pressure via a G_i-dependent mechanism, with males having higher blood pressure than females (Keys et al., 2005). Overexpression of GRK2 increased the thickness of vesicular smooth muscle, suggesting that overexpression of GRK2 is sufficient to induce cardiac hypertrophy (Eckhart et al., 2002). This effect might contribute to hypertension by narrowing the blood vessel lumen due to increased wall thickness. In contrast to GRK2, overexpression of GRK5 did not cause vascular or cardiac hypertrophy (Keys et al., 2005). The role of GRK3, which is also expressed in vesicular smooth muscle, in hypertension remains essentially unexplored. Inhibition of GRK3 in the heart causes hypertension due to increased cardiac output attributed to increased sensitivity of cardiac α1B-adrenoreceptors (Vinge et al., 2008).

Kidneys control water and sodium content in the blood, thus playing an important role in long-term maintenance of normal blood pressure. Dopamine regulates water and electrolyte transport in renal proximal tubules via D1 dopamine receptors, and D1 receptor stimulation invokes natriuretic response. In contrast, dopamine fails to induce natriuresis in patients with hypertension (O'Connell et al., 1997) and in animals with essential hypertension (Nishi et al., 1993). In hypertension, the function of D1 receptors is impaired: the D1 receptor-mediated cAMP production is reduced, the receptor phosphorylation is increased, the receptor is internalized, and its recycling back to the plasma membrane is inhibited (Sanada et al., 1999; Sanada et al., 2006b; Yu et al., 2006). In renal proximal tubules, D1 receptors are desensitized by GRK4, with GRK2 playing a less prominent role (Watanabe et al., 2002). In spontaneously hypertensive rats, the basal expression of GRK4 is increased (Sanada et al., 2006b). Single nucleotide polymorphisms R65L, A142V, and A486V leading to increased kinase activity of GRK4γ splicevariant and uncoupling of the D1 dopamine receptor from its cognate G proteins has been positively linked to essential hypertension (Felder et al., 2002). The same variants were associated with salt-sensitive hypertension (when blood pressure increased more than 10% with increased sodium intake), and the frequency of GRK4 variants was higher in salt sensitive than in salt-resistant hypertensive patients (Sanada et al., 2006a). Transgenic mice overexpressing A142V variant have higher blood pressure than wild type mice or mice overexpressing wild type GRK4γ (Felder et al., 2002). Mice overexpressing A486V variant displayed salt-sensitive hypertension (Felder and Jose, 2006). Reducing the expression of GRK4 with antisense oligonucleotide infusion increased sodium excretion and ameliorated hypertension in spontaneously hypertensive rats (Sanada et al., 2006b; Yatabe et al., 2008). It should be noted, however, that later studies failed to fully replicate the initial association data by Felder and colleagues, with only A486V variant showing robust association with hypertension in human patients (Bengra et al., 2002; Speirs et al., 2004; Wang et al., 2006; Martinez Cantarin et al., 2010). Furthermore, wild type GRK4γ so far has shown little activity in regulating GPCRs (Premont et al., 1999; Felder et al., 2002). This splice variant is missing 15^{th} exon corresponding to 46 amino acids of the second half of α helix 10 and all of α helix 11 of the N-terminal portion of RH domain, and, based on the existing structure, it might have stability and/or folding problems (Lodowski et al., 2006). This is an exciting area of research with obvious therapeutic potential. More extensive structural and functional information on GRK4 and its splice variants would be of great value.

A related issue is dysregulation of renal dopamine D1 receptors in the condition of insulin resistance and the role of GRKs in that process. Obesity-induced insulin resistance impairs the function of D1 dopamine receptors in renal proximal tubules leading to reduced sodium excretion and hypertension. In this case GRK4 also appears to be the main culprit, with some contribution of GRK2. The expression of GRK4 and, to a lesser extent, GRK2 is elevated in the renal proximal tubules of obese animals (Umrani et al., 2002) leading to hyper-phosphorylation of D1 dopamine receptor and its uncoupling from G_s (Hussain et al., 1999; Trivedi and Lokhandwala, 2005). Treatments with insulin-sensitizing drug rosiglitazone restored D1 receptor coupling to G_s, decreased D1 receptor phosphorylation and selectively decreased the expression of GRK isoforms in the renal proximal tubules of obese but not lean rats (Trivedi and Lokhandwala, 2005). Although GRK2 might be less important than GRK4 for the renal D1 receptor desensitization, blocking GRK2 membrane translocation in insulin-treated kidney cells or reducing GRK2 expression with siRNA alleviated D1 receptor phosphorylation and normalized D1 expression and G_s coupling (Banday et al., 2007).

GRK2 has a role in the development of insulin resistance via its control of the function of βARs. βARs are universal mediators of the regulatory influence of the sympathetic nervous system on multiple metabolic pathways, including lipid and lipoprotein metabolism and

glucose homeostasis in adipose tissue, liver, and skeletal muscle. Insulin resistance related to abnormalities in insulin signaling is common in diseases that increase cardiovascular risk and mortality such as diabetes, obesity, hypertension, and heart failure. Enhanced sympathetic nervous system signaling, which is typical of several cardiovascular diseases, plays a role in the pathogenesis of insulin resistance, because βAR stimulation results in impairment of insulin signaling both in adipocytes and cardiac myocytes, albeit in a tissue-specific manner (Morisco et al., 2006). Increased βAR signaling induced GRK2 accumulation in cultured cells accompanied by a deficit in insulin-induced glucose uptake. Inhibition of GRK2 led to increased insulin sensitivity both in cultured cells and in spontaneously hypertensive rats, an animal model of insulin resistance (Cipolletta et al., 2009). Data using GRK2 hemizygous mice, as well as cultured myoblasts and adipocytes with GRK2 levels elevated or reduced, demonstrated that lower GRK2 level induces enhanced insulin sensitivity, lean phenotype and protects against TNFα-, high fat diet-, or aging-induced insulin resistance (Garcia-Guerra et al., 2010). Importantly, GRK2 can directly inhibit the function of the insulin-responsive glucose transporter 4 by sequestering $G_{q/11}$ via its RGS domain and thus inhibiting $G_{q/11}$–mediated signaling (Usui et al., 2004). GRK2 can also contribute to insulin resistance by inhibiting insulin signaling via phosphorylation of IRS- in response to stimulation of GPCRs such as endothelin-A receptor (Usui et al., 2005). Taken together, these data suggest that GRK2 is central for the development of insulin resistance and is a viable target for therapy.

9.3. GRK function in the immune system

GPCRs mediate the activity of many modulators of the immune process such as chemokines, leukotrienes, and neurotransmitters. Thus, it is not surprising that GRKs, which are the main regulators of the GPCR activity, turned out to be involved in many aspects of the inflammation process. The expression of multiple GRK isoforms has been shown to alter in human immune diseases and in animal models. GRK2 concentration is reduced in peripheral blood mononuclear cells of patients with active relapsing-remitting multiple sclerosis (MS) or with secondary progressive MS (Giorelli et al., 2004; Vroon et al., 2005). The expression of GRKs 2 and 6 was reduced in splenocytes, but not in the heart, liver, or pituitary, in rats with experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Vroon et al., 2003). The levels of GRKs 2 and 6, but not that of GRK5, were also decreased in lymphocytes of patients with rheumatoid arthritis (Lombardi et al., 1999), and in immune cells in the adjuvant animal model of arthritis (Lombardi et al., 2001). The concentration of GRK2 was elevated in neutrophils from patients with sepsis, as well as in control neutrophils treated with cytokines plus lipopolysaccharide (to mimic septic environment) that might result in septic neutrophils' insensitivity to chemoattractants (Arraes et al., 2006). Neonatal experimental hypoxia/ischemia in rats decreased the expression of GRK2, but not GRK6, in the ipsilateral hippocampus that suffered severe damage following the injury (Lombardi et al., 2004).

The functional consequences of changes in the GRK availability for the course of the immune process were highlighted by studies with GRK knockout mice. Loss of GRK2 in GRK2 hemizygous mice induced earlier onset of experimental autoimmune encephalomyelitis, an animal model of MS, although GRK2+/− mice, in contrast to wild type, only developed an acute phase of the disease without relapses (Vroon et al., 2005). The acute inflammation response (ear swelling and infiltration of polymorphonuclear neutrophils into the ear) following topical application of arachidonic acid was markedly increased in GRK6 knockout mice due to elevated signaling via the leukotriene B_4 receptor BLT1 (Kavelaars et al., 2003). Loss of GRK6 reduced migration of neutrophils out of the bone marrow in response to granulocyte-colony stimulating factor (G-CSF), likely because of increased retention of neutrophils in the bone marrow resulted from enhanced signaling via

the stromal cell-derived factor-1/CXC chemokine receptor 4 pathway, which is the main retention signal for neutrophils (Vroon et al., 2004b). Loss of GRK6 increased the severity of experimental colitis in GRK6−/ − and GRK6+/− mice, which was accompanied by increased keratinocyte-derived chemokine level and increased granulocyte infiltration (Eijkelkamp et al., 2007). Furthermore, wild type mice completely recovered from colitis, whereas GRK6 knockout and hemizygous mice developed chronic colitis. In the neonatal hypoxia/ischemia model, loss of GRK2 lead to aggravated white and grey matter brain damage and enhanced cerebral neutrophil infiltration (Nijboer et al., 2008). Selective deletion of GRK2 in microglia/macrophages was sufficient to induce early onset of the injury, possibly due to accelerated microglia activation, whereas selective deletion of GRK2 in neurons augmented the severity of the brain damage without affecting the onset (Nijboer et al., 2010).

GRKs are known to regulate the signaling via multiple chemokine receptors, which, as GPCRs, are subject to GRK-dependent phosphorylation and desensitization (Aramori et al., 1997; Aragay et al., 1998; Vroon et al., 2004a). Additionally, GRKs may affect chemokine signaling via direct interaction with signaling molecules such as MEK (Jiménez-Sainz et al., 2006). In their turn, inflammatory mediators have been shown to regulate the expression of GRKs in immune and other cells via a variety of mechanisms, such as signaling (Fan and Malik, 2003; Lombardi et al., 2007), transcription (Ramos-Ruiz et al., 2000; Fan and Malik, 2003), or degradation (Lombardi et al., 2002; Cobelens et al., 2007), all of which might contribute to alterations in GRK expression in diseases.

There is still a long way to go before the precise functional role of each GRK isoform in various categories of inflammation processes are fully understood. The complexity of the immune system and regulatory mechanisms involved make this a formidable task.

9.4. GRK function in the brain

Four out of five non-visual GRK isoforms, GRKs 2, 3, 5, and 6, are highly expressed in the brain (Gurevich et al., 2004; Bezard et al., 2005; Ahmed et al., 2007; Bychkov et al., 2008; Bychkov et al., 2010; Bychkov et al., 2011). The GRK concentration in the brain increases between birth and adulthood, with GRK5 being the only isoform that peaks at birth and declines afterwards (Gurevich et al., 2004). In the adult rat brain, GRK6 is the main isoform followed by GRK2, with GRKs 3 and 5 expressed at lower levels (Gurevich et al., 2004; Ahmed et al., 2007). In contrast, GRKs 5 and 2 are the main isoforms in the primate brain, with GRKs 6 and 3 expressed at lower levels (Bezard et al., 2005; Bychkov et al., 2008). GRK3 is the least abundant in rodent and primate subcortical brain areas, but its expression in the cortex is comparable to that of other isoforms (Bezard et al., 2005; Ahmed et al., 2007; Bychkov et al., 2008). GRK4 has a restricted expression, but it is expressed in cerebellar Purkinje cells, where it mediates desensitization of the metabotropic glutamate receptor 1 (Sallese et al., 2000b). Neurons, the cells with the most intense and sophisticated signaling, express multiple GPCRs. The mammalian GRK3 has been assigned at least one specialized neural function: it mediates desensitization of odorant receptors in the olfactory epithelium (Dawson et al., 1993; Peppel et al., 1997). Interestingly, when GRK3 gene is disrupted, not only receptor desensitization is impaired, but also the odorant-induced cAMP production suggesting a signaling role for GRK3 (Peppel et al., 1997). Similarly, in *C. elegans*, Grk2 (also referred to as Gprk, in GRK2/3 subfamily) is required for normal chemosensation (Fukuto et al., 2004). In *Drosophila*, Gprk2 (in GRK4/5/6 subfamily) is required for circadian odorant responses (Tanoue et al., 2008). Therefore, it seems evident that GRKs play critical roles in the brain functions, be it via the GPCR desensitization mechanisms or via their signaling functions. Dysfunction of GRK isoforms has been implicated in several neurological and psychiatric conditions.

9.4.1. GRKs in Alzheimer's disease—Abnormalities of the brain vascular system make a critical contribution to the pathogenesis of Alzheimer's disease (AD). As discussed above, GRK2 is heavily involved in the regulation of vascular tone and blood pressure via its action at GPCRs expressed by smooth muscle and endothelial cells of the blood vessels. GRK2 was upregulated in endothelial cells in AD patients and in the two-vessel carotid artery occlusion model, which mimics vascular changes in early AD (Obrenovich et al., 2006). AD patients showed elevated expression of GRK2 protein and mRNA, and the level correlated with the degree of cognitive impairment (Leosco et al., 2007). In early onset AD transgenic mouse model, the expression of GRK2, but not GRK5, in the cortex was increased. However, the membrane fractions of both GRKs 2 and 5 were reduced in transgenic mice, as compared to wild type (Suo et al., 2004). Both changes occurred before cognitive decline and were likely linked to the effect of sub-threshold soluble β-amyloid. Loss of active GRKs resulted in impaired receptor desensitization and enhanced signaling. The role of GRK5 in cognition is underscored by evidence that aged GRK5 knockout mice display working memory deficits. Pathological changes in the hippocampal neurons were noted in these mice, including axonal swellings, depositions of structural proteins, even β-amyloid deposits, along with loss of synaptic proteins (Suo et al., 2007; Li et al., 2009). GRK5 deficiency also augmented inflammation in TgAPPsw (Tg2576) transgenic mice increasing both microgliosis and astrogliosis in the cortex and hippocampus (Li et al., 2008). Deficient GRK5 activity accelerates accumulation of β-amyloid, as reflected in increased plaque burden and increased level of soluble β-amyloid, in the cortex of Tg2576 mice (Cheng et al., 2010b). The effect was due to hyperactivity of presynaptic M2 autoreceptors and reduced acetylcholine release (Liu et al., 2009; Cheng et al., 2010b). Therefore, β-amyloid-induced deficit of membrane-bound GRK5 propagates the pathology by facilitating further accumulation of β-amyloid, promoting inflammation, neuronal damage, and cognitive impairment. A better mechanistic understanding of how β-amyloid causes translocation of GRK5 from the membrane to the cytosol is a prerequisite for a viable therapeutic approach in AD directed at GRK5. Furthermore, the ability of β-amyloid to suppress membrane localization of GRK2, which has a different mechanism of membrane recruitment than that of GRK5, while increasing its overall expression, should also be explored.

9.4.2. GRKs in Parkinson's disease and L-DOPA-induced dyskinesia—Parkinson's disease (PD) is an age-related movement disorder caused by the loss of dopaminergic neurons in the substantia nigra pars compacta that provide dopaminergic innervation to the striatum, the structure heavily involved in the control of the movement. Loss of dopamine (DA) in the striatum induces multiple alterations in the signaling via dopamine receptors expressed by striatal neurons [reviewed in (Gurevich and Gurevich, 2010a)]. In the rat 6-hydroxydopamine (6-OHDA) model of PD, loss of DA causes downregulation of most GRK isoforms in the motor part of the striatum (Ahmed et al., 2007; Ahmed et al., 2010), which could be interpreted as a compensatory response designed to slow down desensitization of DA receptors to keep up their responses in the face of DA depletion. A tendency to a similar downregulation was seen in the striatum of cognitively intact human patients with PD at postmortem, although the effect did not reach significance (Bychkov et al., 2008). In contract, there was no reduction in the expression of GRKs in the primate model of PD (non-human primates lesioned with dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropydridine, MPTP), and the expression of GRK6 was even significantly elevated (Bezard et al., 2005), which is hard to explain mechanistically. The main treatment for PD today is symptomatic, aimed at restoring DA to the brain using a DA precursor L-DOPA. L-DOPA therapy is quite successful for several years, but after that the drug loses effectiveness, and a number of side effects emerge, including L-DOPA-induced dyskinesia (LID) (Fahn, 2008). LID is easily modeled in rodents and primates. In the 6-OHDA-lesioned rats, L-DOPA produces rotations contralateral to the side of the lesion, and

the rotation frequency increases with chronic treatment. This increase is known as behavioral sensitization to L-DOPA and is considered an animal model of LID. Despite decades of intense studies, molecular mechanism of LID remains poorly understood [reviewed in (Gurevich and Gurevich, 2010a)]. Chronic L-DOPA treatment in 6-OHDA-lesioned rats did not restore reduced levels of GRK expression, whereas a long-lived DA agonist pergolide, which was less potent than L-DOPA in inducing dyskinesia, had a tendency to do so (Ahmed et al., 2007). In MPTP monkeys treated chronically with L-DOPA, manifestations of LID are similar to those observed in human PD patients. Interestingly, in monkeys chronically treated with L-DOPA the levels of GRKs 2 and 6 in the striatal regions were lower than in drug-naïve parkinsonian monkeys (Bezard et al., 2005), suggesting that chronic L-DOPA suppresses the GRK expression. In human PD patients, the level of GRKs tended to be lower than in controls (Bychkov et al., 2008), but since human PD patients are routinely treated with L-DOPA, it is impossible to separate the effects of PD itself from those of L-DOPA treatment. To summarize, the data on the changes in the expression of GRKs in PD are inconsistent across animal models of PD and human studies. The rat data make the most mechanistic sense.

What is undisputed, however, is that DA receptors in the DA-depleted striatum are supersensitive to dopaminergic stimulation. This supersensitivity manifests itself in exaggerated responsiveness of a multitude of signaling pathways, as well as in stronger behavioral responses and, paradoxically, seems to be further aggravated in LID [reviewed in (Gurevich and Gurevich, 2010a)]. The supersensitivity of the D1 and D3 DA receptors and impaired D1 receptor trafficking (Guigoni et al., 2007; Berthet et al., 2009) have been implicated in LID (Bezard et al., 2003; Aubert et al., 2005). The molecular underpinnings of the supersensitivity remain unclear. One possible mechanism that could result in elevated responsiveness to stimulation is impaired desensitization due to deficiency of the GRK-dependent receptor phosphorylation and subsequent arrestin binding. A defect in the phosphorylation step is quite likely to be of consequence, since it appears to be rate-limiting for the desensitization process (Violin et al., 2008). Therefore, increasing availability of GRKs might promote desensitization of DA receptors, reduce signaling, and alleviate LID. The search for anti-LID therapy has been challenging. The goal is to preserve just enough of dopaminergic signaling, otherwise PD patients would revert to the akinetic/bradykinetic state, whereas excessive signaling responsible for LID should be suppressed. In practice, however, it has been difficult to impossible to reduce LID without diminishing the “good” effect of the drug, because the mechanisms of LID and those of the antiparkinsonian effect of L-DOPA are so intertwined. From mechanistic standpoint, it is reasonable to expect that GRK therapy can offer new hope due to exquisite selectivity of GRKs for active receptors. The dopamine receptors should be activated and some signaling will go through before the receptors are phosphorylated by GRKs and desensitized, thus allowing for therapeutic activity of L-DOPA but preventing LID. Generally speaking, GRKs are among a few molecular tools that can achieve this balance, as this is their intended function in cells. Upon activation, the receptor first encounters several G protein molecules, thereby transducing the signal, before GRKs have a chance to attach sufficient number of phosphates [usually 2–3 (Gurevich et al., 1995; Vishnivetskiy et al., 2007)] to promote high-affinity binding of arrestins, which cover the cytoplasmic tip of the receptor, sterically precluding further G protein activation (Wilden, 1995; Krupnick et al., 1997). Thus, classical two-step mechanism of receptor shutoff provides the time window necessary for the signal to go through before G protein activation is blocked, preventing excessive signaling. The idea of taking advantage of this mechanism has been tested in the rodent and primate models of PD using lentivirus-mediate overexpression of GRK6, the kinase isoform shown to be most relevant for the DA receptors *in vivo* (Gainetdinov et al., 2003). Increased level of GRK6 in lesioned rat striatum significantly reduced the number of contra-lateral rotations and abnormal involuntary movements induced by L-DOPA, indicating that extra GRK6

suppresses LID (Ahmed et al., 2010). The findings in MPTP-lesioned monkey were even more promising. As mentioned earlier, the main difficulty in developing a viable anti-dyskinetic therapy is to provide relief from dyskinesia without losing beneficial antiparkinsonian efficacy of a drug. Interestingly, in dyskinetic monkeys overexpression of GRK6 alleviated LID and at the same time extended the antiparkinsonian action of L-DOPA (Ahmed et al., 2010). The effect was particularly striking with half-dose of L-DOPA. GRK6-expressing monkeys on half-dose of L-DOPA had the same duration of L-DOPA antiparkinsonian activity as control monkeys on full dose of L-DOPA. Conversely, GRK6 knockdown exacerbates dyskinesia in rodent and primate models of Parkinson's disease, suggesting that GRK6-mediated regulation of the dopamine receptor signaling is involved in dyskinesia (Ahmed et al., 2010). This study is a good example of how the mechanistic understanding of the GRK function can be successfully translated into a promising therapeutic application.

As discussed in Section 5, GRK5 phosphorylates α-synuclein (Pronin et al., 2000). α-Synuclein is the main component of Lewy bodies found in sporadic Parkinson's disease, as well as other synucleopathies. α-Synuclein accumulated in Lewy bodies has often undergone posttranslational modifications, such as phosphorylation. GRK5 has been identified as a kinase that phosphorylates α-synuclein, promotes its oligomerization, and co-localizes with it to Lewy bodies in the substantia nigra and locus coeruleus of patients with PD, but not in the cortex of patients with Lewy body disorder (LBD) (Arawaka et al., 2006). Furthermore, two single nucleotide polymorphisms enhancing promoter activity found in introns of GRK5 were associated with the risk of sporadic Parkinson's disease (Arawaka et al., 2006). However, this finding created some controversy, because later studies failed to reproduce localization of GRK5 to Lewy bodies (Takahashi et al., 2006) or association of polymorphisms in GRK5 gene and Parkinson's disease (Tarantino et al., 2011). Furthermore, knockdown of endogenous GRK5 failed to diminish phosphorylation of α-synuclein in cultured cells (Sakamoto et al., 2009; Liu et al., 2010). GRKs 3 and 5 were strongly upregulated at postmortem in the striatal regions of the PD patients with dementia, but not in cognitively intact patients (Bychkov et al., 2008). GRK2 was associated with neurofibrillary tangles in the brains of patients with Alzheimer's disease (AD) and AD combined with LBD (Takahashi et al., 2006). GRK2 was also found in tau fibrillary deposits in other disorders, such as progressive supranuclear palsy, corticobasal degeneration, and Pick's disease (Takahashi et al., 2006). These data, although requiring confirmation, suggest that increased concentration of GRK isoforms in PD may not be entirely a good thing and argue for caution in targeting GRKs for anti-LID therapy. Importantly, GRK6 has never been implicated in adverse effects in PD.

9.4.3. GRKs in depression—Major depression is a severe psychiatric disorder of complex etiology associated with multiple plastic molecular changes in the neural networks (reviewed in (Krishnan and Nestler, 2010)). Antidepressant drugs acting via a variety of mechanisms improve mood in the disorder. The activity of multiple GPCRs is altered in the depressive state and affected by antidepressant treatment (Catapano and Manji, 2007; Dunlop and Nemeroff, 2007). GRKs as major regulators of the efficacy of the GPCR signaling have been implicated in the mechanisms of depression and actions of antidepressant drugs. The concentration of membrane-associated GRKs 2 and 3 was increased in the prefrontal cortex of depressed suicide victims at postmortem (García-Sevilla et al., 1999), and this upregulation was normalized by antidepressant treatment (Grange-Midroit et al., 2003). In contrast, GRK6 was not affected (Grange-Midroit et al., 2003). The function of GPCRs in major depression are altered not only in the brain but also in blood cells in a manner associated with the clinical status and action of psychotropic drugs. Due to easy availability of blood samples from living patients (in contrast to the brain), these changes have often been used as biomarkers for pathological changes in psychiatric

disorders (Elliott, 1992; Camacho and Dimsdale, 2000). The concentration of GRK2, but not GRK6, in platelets in depressed patients was decreased in parallel with the upregulation of α2A-adrenoreceptors, and the severity of depression in drug-free patients inversely correlated with the GRK2 platelet concentration (García-Sevilla et al., 2004). Treatment with antidepressant mirtazapine reduced the α2A-adrenoreceptor concentration and restored the level of GRK2. Similarly, GRK2 protein and mRNA were significantly reduced in mononuclear leukocytes in untreated patients with major depression as compared to healthy controls, and this downregulation was ameliorated by antidepressant treatment (Matuzany-Ruban et al., 2010). Moreover, the increase in the GRK2 level preceded the clinical improvement and was predictive of it, making GRK2 a biomarker for antidepressant effect. Furthermore, upregulation of GRK2 in platelets of patients with major depression induced by selective serotonin reuptake inhibitor (SSRI) antidepressants discriminated between responder and non-responder patients (García-Sevilla et al., 2010). GRK3 also showed a modest reduction in platelets in depressed subjects, whereas GRK5 was unchanged. There was no change in GRK2 or GRK5 mRNA in lymphocytes of untreated depressed subjects but antidepressants upregulated GRK2 mRNA (García-Sevilla et al., 2010).

Two independent genome-wide linkage analysis studies have suggested a location for a susceptibility locus for bipolar disorder on chromosome 22q12 around the microsatellite markers D22S419 and D22S533 (Edenberg et al., 1997; Kelsoe et al., 2001). These markers are in the immediate vicinity of the GRK3 gene. A single nucleotide polymorphism in the GRK3 promoter that enhances the GRK3 gene expression has been shown to associate with major depression (Barrett et al., 2003; Barrett et al., 2007; Zhou et al., 2008) suggesting that increased expression of GRK3 in select brain regions might be involved in depression, which is generally in agreement with the data showing increased expression of GRK2/3 in the brain of depressive subjects (García-Sevilla et al., 1999; Grange-Midroit et al., 2003). The mechanism by which the GRK3 elevation translates into depression and which GPCRs might be involved is unclear. Some possibilities are suggested, however, by animal studies. Down-regulation of GRK3 in the nucleus accumbens has been shown to be associated with increased sensitivity to reward (Dinieri et al., 2009). GRK3 is also involved in mediating stress-induced depression (repeated swim-induced immobility) or dysphoric effect of κ-opioid receptor stimulation (Bruchas et al., 2007). The stress effect is dependent on the activation of κ-opioid receptor and on activation of p38 mitogen-activated protein kinase in the nucleus accumbens. The role of GRK3 is likely to facilitate the recruitment of arrestins to κ-opioid receptors, since κ-opioid receptor-induced p38 activation is GRK3- and arrestin-dependent (Bruchas et al., 2006). Learned helplessness, a behavior considered an animal model of depression (Vollmayr and Henn, 2001), is associated with reduced GRK3 expression in the locus coeruleus and amygdala in rats, whereas rats that do not develop learned helplessness following forced swim have normal GRK3 expression (Taneja et al., 2011). This deficit in the GRK3 expression seems linked to the dysregulation of the α2-adrenoreceptors and corticotropin releasing factor 1 receptors in rats with learned helplessness. To conclude, there is sufficient evidence that GRKs 2 and 3 are involved in the mood disorders, and it appears likely that upregulation of both kinases in the brain, particularly in the prefrontal cortex and nucleus accumbens, is associated with depression. So far, the members of the GRK4 subfamily have not been implicated in the mood disorders.

9.4.4. GRKs in pain—Chronic pain is a huge clinical problem that is still poorly managed. Neuropathic pain is chronic pain resulting from damage to the somatosensory system either centrally or peripherally. Many patients with neuropathic pain manifest hyperalgesia (enhanced sensitivity to nociceptive stimuli) or allodynia (painful response to non-nociceptive stimuli) [for review see (Baron et al., 2010)]. In rats with experimentally induced allodynia (by chronic constriction injury to sciatic nerve), the expression of GRK2 in the superficial layers of the dorsal horn of the lumbar spinal cord was decreased

(Kleibeuker et al., 2007), which was likely mediated by inerleukin-1 β signaling (Kleibeuker et al., 2008). Specifically, there was a reduction in the GRK2 level in spinal microglia/macrophages associated with mechanical allodynia (Eijkelkamp et al., 2010b). Furthermore, reduction in GRK2 concentration in GRK2 hemizygous mice lead to enhanced mechanical allodynia (Kleibeuker et al., 2007).

Chronic pain associated with inflammatory process is a common clinical problem. Inflammation leads to hyperalgesia and allodynia due to increased excitability of peripheral nociceptive sensory nerves caused by the action of inflammatory mediators including cytokines, chemokines, peptides, and neurotransmitters, among many others [for review see (Linley et al., 2010)]. Since many of these agents act via GPCRs, it should come as no surprise that GRKs are involved in modulating inflammatory pain. Animals with experimentally induced chronic paw inflammation (injection of a dose of carrageenan) show ~35% reduction in the level of GRK2 in small diameter sensory neurons in the dorsal root ganglia (Eijkelkamp et al., 2010a) and ~40% reduction in microglia/macrophages (Willemen et al., 2010). GRK2 hemizygous mice with reduced level of GRK2 developed more intense and prolonged thermal hyperalgesia and mechanical allodynia following experimental paw inflammation (Eijkelkamp et al., 2010b). When GRK2 was selectively knocked down in peripheral sensory nociceptive neurons, carrageenan- and chemokine CCL3-induced hyperalgesia was enhanced but nor prolonged, whereas selective knockdown of GRK2 in microglia/macrophages caused chronic hyperalgesia in response to carrageenan, chemokine CCL3 (Eijkelkamp et al., 2010b), and interleukin-1 β (Willemen et al., 2010) due to sustained microglia activation. In contrast, selective neuronal knockdown of GRK2 markedly prolonged hyperalgesia caused by prostaglandin E_2 or 8-Br-cAMP via Epac/PKCε/ERK-dependent pathway (Eijkelkamp et al., 2010a). These data point to GRKs, GRK2 isoform in particular, as major mediators of chronic pain. The evidence suggests that reduced concentration of GRK2 might enhance pain via deficient desensitization and resulting supersensitivity of receptors for inflammatory mediators, as well as via deficits in GRK-dependent signaling pathways.

10. Conclusions and future prospects

GRKs are well-established regulators of signaling and trafficking of GPCRs, the most numerous class of cell surface receptors targeted by about half of clinically used drugs (Jacoby et al., 2006). Recent findings show that GRKs also regulate a number of other GPCR-independent signaling pathways intimately involved in many vital cellular functions. Despite their obvious importance, GRKs are under-appreciated as drug targets and therapeutic tools.

In order to take full advantage of GRKs as therapeutic targets, novel and diverse therapeutic approaches are likely to be necessary. The biggest advantage of GRKs is that they act essentially as receptor activation sensors modulating the receptor signaling according to a current physiological context. Conventional small molecule drugs, such as receptor agonists or antagonists, or enzyme inhibitors, perform a single function of activation or inhibition regardless of the functional state of the patient. They cannot receive feedback from the body, and therefore can easily overdo whatever they are designed to do, often turning therapeutic action into harm. However, small molecule drugs do have distinct advantages such as relative ease of development, production, and delivery, as well as established methods of optimizing metabolism and achieving acceptable pharmacokinetics. Since the discovery of GRKs, attempts have been made to find their inhibitors to use as research tools. Furthermore, a considerable effort has been put into the development of selective GRK inhibitors, particularly that of GRK2 to be used in heart failure therapy. These efforts have met with modest success. Polyanionic compounds heparin and dextran sulfate that potently

inhibit GRK2 lack selectivity (Benovic et al., 1989a), and so does a natural compound balanol, a potent inhibitor of AGC kinases (Setyawan et al., 1999; Tesmer et al., 2010), whereas others lack potency or have non-drug-like properties (e.g. peptides), or all of the above (Iino et al., 2002; Winstel et al., 2005). Recently, Takeda Pharmaceuticals developed potent inhibitors selective for the GRK2/3 subfamily (Ikeda et al., 2007; Thal et al., 2011) that might prove useful for the therapy of cardiovascular diseases, but no additional information is available. So far, no inhibitors selective for the kinases of GRK4/5/6 subfamily have been developed. Further elucidation of the molecular mechanisms of GRK regulation and high-resolution structures of their catalytic sites will allow the development of a wide array of isoform-specific inhibitors, which would be useful in situations when the activity of a particular isoform needs to be dampened for therapeutic purposes (Fig. 2) (Rockman et al., 1998b; White et al., 2000; Harding et al., 2001; Shah et al., 2001; Rengo et al., 2009). Alternatively, developing practical methods of *in vivo* GRK knockdown in various tissues with shRNA or microRNA might provide an advantage over GRK inhibitors in isoforms specificity and in that the responsiveness of a GRK to the physiological environment would be preserved.

In addition to or instead of a straightforward inhibition of GRK activity, a finer modulation of GRK activity might also be attempted. As discussed above, in many cases GRK modulate the GPCR signaling in a phosphorylation-independent manner. The physiological significance of this mode of regulation is less studied than that of ‘classic” phosphorylation-mediated receptor desensitization, and the role of phosphorylation-independent regulation of signaling by GRKs in diseases remains essentially unknown. However, these interactions might also be targeted to achieve desired modulation of receptor signaling. GRK interactions with the α subunits of G_q subfamily, Gβγ, and MAP kinases can be modulated by conventional small molecules, especially when high-resolution structures of multi-protein complexes allow targeted drug design, although the targeting of protein-protein interfaces remains challenging.

A pilot study shows that GRKs are promising molecular tools for the therapy of certain neurological and, perhaps, psychiatric disorders (Ahmed et al., 2010) (Fig. 3). The major difficulty in this case is that *enhanced* GRK activity is required (Fig. 3), perhaps, even the enhanced activity of a particular GRK isoform, GRK6 (Ahmed et al., 2010). Currently, there is no way to elevate the GRK function except by overexpression via gene therapy. It is possible that for many situations gene therapy will remain the only option for a foreseeable time. Gene therapy is a legitimate therapeutic tool and should be developed with improved delivery systems and a wide array of cell-specific promoters that could be turned on and off by small molecules, as needed. An advantage of gene therapy is that it does not have to be limited to wild type proteins. Mutant GRKs with enhanced receptor selectivity, enhanced kinase activity, or any other required modification could also be employed. However, as a prerequisite, the mechanisms of the regulation of normal signaling in the cell must be elucidated at fine molecular level to enable the design of optimally modified GRK proteins to be delivered. Alternatively, small molecules enhancing or reducing transcription and/or degradation of individual GRK isoforms and splice variants can be designed to increase or decrease their endogenous expression, as needed. This development requires detailed information on the regulation of GRK synthesis and degradation in the cell at the molecular and structural level that is absent at this time. Modulating the GRK concentration via overexpression or control over degradation may be the only way to fine-tune an overactive signaling pathway and retain its full responsiveness to dynamic physiological regulation at the same time, in order to cure a disease rather than just treat the symptoms. As therapeutic tools, proteins offer endless possibilities that need to be explored and exploited.

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Abbreviations

AD	Alzheimer's disease
β2AR	β_2-adrenergic receptor
CDK2	cyclin-dependent kinase 2
GAP	GTPase activating proteins
GIRK	G protein-coupled potassium channels
GIT	GRK interacting proteins
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HDAC	histone deacetylase
IRS	insulin receptor substrate
KD	kinase domain
LBD	Lewy body disorder
LID	L-DOPA-induced dyskinesia
MS	multiple sclerosis
PD	Parkinson's disease
PDK1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PI3K	phosphoinosidite-3-kinase
RGS	regulator of G protein signaling
RH	RGS homology
S1P	sphingosine-1-phosphate
SSRI	selective serotonin reuptake inhibitor
Smo	Smoothened
Ptc	Patched
Hh	hedgehog

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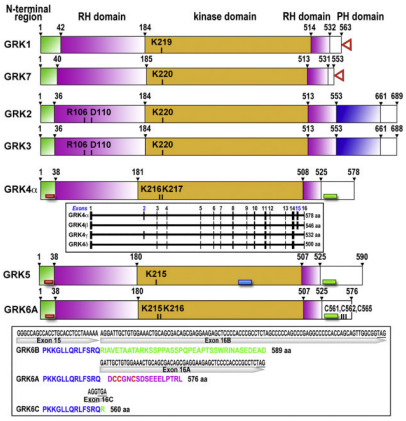

Figure 1. Domain structure of GRKs

Number above the structures indicate amino acid residue numbers of human GRKs based on (Lodowski et al., 2006). All GRKs have a short N-terminal region (green), which is implicated in GPCR binding, followed by RGS homology (RH) domain (magenta). This N-terminal region is unique to the GRK family of kinases. The RH domain is interrupted by the catalytic domain shared by all kinases (dark yellow). These elements are shared by the GRK2/3 and GRK4/5/6 subfamilies. The defining feature of the GRK2/3 subfamily is a C-terminal pleckstrin homology (PH) domain (blue) implicated in binding anionic phospholipids and Gβγ. Members of GRK4/5/6 subfamily use alternative mechanisms for membrane targeting, which include palmitoylation [palmitoylation sites are shown for GRK6A (Jiang et al., 2007)], patches of positively charged residues [amphipathic helix motifs (Thiyagarajan et al., 2004; Jiang et al., 2007) are shown as green boxes; N-terminal basic patches (Pitcher et al., 1996; Boguth et al., 2010) are shown as red boxes], and, in case of visual subtypes, prenylation (C-terminal prenylation sites in GRK1 and 7 are shown as red triangles). Residues Arg106 and Asp110 in GRK2/3, among others, are important for binding $G\alpha_q$, a function unique to this subfamily. The position of the key lysine responsible for catalysis in the kinase domain is shown. Mutations K220R in GRK2 and 3, as well as K216M/K217M (Sallese et al., 2000b) in GRK4, K415 in GRK5 (Tiruppathi et al., 2000), and K215M/K216M in GRK 6(Lazari et al., 1999) yield kinase-dead GRKs. The blue box shows the position of the nuclear localization signal (NLS) in GRK5 (residues 388–395) (Johnson et al., 2004). Splice variants of GRK 4 (GRK4β, GRK4γ, and GRK4δ) are produced by in-frame deletion of exon 2 (GRK4β), exon 15 (GRK4γ), or both (GRK4δ (Premont et al., 1996; Sallese et al., 1997; Premont et al., 1999) (gene structure is shown under GRK4 protein; the exons not used in all splice variants are shown in blue) GRK6 splice variants are produced by a frame shift in the C-terminus resulting in a completely different C-terminal sequence in GRK6B as compared to GRK6A and in premature transcription termination in GRK6C (Premont et al., 1999). To generate GRK6A, exon 16 starts two nucleotides downstream, as compared to the longest variant GRK6B, resulting in a frame shif. An alternative upstream exon 16 encoding one amino acid before the stop codon is used to generate GRK6C (respective exons are labeled Exon 16A, Exon 16B, and Exon 16C). In the C-termini of GRK6 splice variants amphipathic helix residues are shown in blue and palmitoylated cysteine in red. Note the lack of palmitoylation sites in GRK6B or GRK6C.

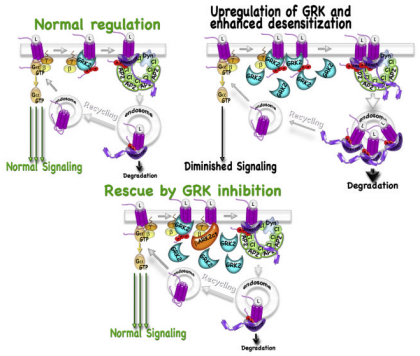

Figure 2. Pathological consequences of enhanced GRK activity and potential therapeutic approaches

Normal regulation of receptor sensitivity involves receptor phosphorylation by a GRK followed by high affinity binding of an arrestin. Arrestin mediates receptor internalization via coated pits followed by either receptor recycling or degradation. In some pathological cases such as heart failure, an upregulation of one or more GRK isoforms can occur. Increased GRK availability leads to receptor hyper-phosphorylation, facilitated receptor desensitization, internalization, and, ultimately, excessive degradation. Similar effect might be achieved when a GRK with a polymorphism yielding higher activity is expressed. Such a pathological phenotype might be rescued by expression of a GRK inhibitor. Here the use of βARKct is shown, which competes with GRK2 and 3 for Gβγ, thus impeding GRK recruitment to active receptors. Alternatively, GRK knockdown could be used.

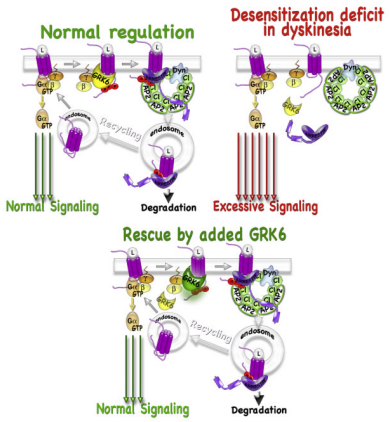

Figure 3. Pathological consequences of reduced GRK activity and potential therapeutic approaches

In some pathological cases exemplified by L-DOPA-induced dyskinesia, a reduced activity of one or more GRK isoforms seems to be the culprit resulting in supersensitivity of dopaminergic receptors to dopamine stimulation. A defect in receptor desensitization and/or trafficking is evidenced by persistence of D1 dopamine receptors at the plasma membrane, whereas in control animals the receptors are internalized upon L-DOPA administration (Guigoni et al., 2007; Ahmed et al., 2010). The deficiency in GRK function need not necessarily be due to reduced expression, but might stem from insufficient GRK availability in the face of increased demand. In case of dyskinesia, human patients with Parkinson's disease or animals with experimental parkinsonism are treated with L-DOPA, which produces surges of dopamine in the brain putting pressure on regulatory signaling mechanisms. Overexpression of an appropriate GRK (e.g., GRK6A was successfully used for L-DOPA-induced dyskinesia (Ahmed et al., 2010)] might relieve that pressure by facilitating receptor desensitization and normal trafficking. Alternatively, positive allosteric modulators of GRKs might be developed. L-DOPA-induced dyskinesia is a good example of a condition where simply shutting down signaling by inhibiting dopamine receptors is not an option, because that would defeat the purpose of L-DOPA treatment, which is to provide dopaminergic stimulation to relieve akinesia in Parkinson's patients. In contrast, using GRK as a target allows for judicial reduction in dopaminergic signaling that alleviates dyskinesia while preserving therapeutic activity of the drug.

Table 1

GRK substrates

Substrate protein	GRK isoform	Tissue/Cells	Function	References
Seven transmembrane domain but non- GPCR receptors				
Smoothened (Smo)	Gprk2 Gprk1	*Drosophila in vivo*	GRK-mediated phosphorylation of Smo is a part of the Smo signaling cascade	(Molnar et al., 2007; Chen et al., 2010; Cheng et al., 2010)
	Grk2, Grk3	zerbrafish *in vivo*		(Philipp et al., 2008)
	GRK2	C3H10T1/2 cells, Shh-LIGHT cells		(Meloni et al., 2006)
Non-GPCR receptors				
Low density lipoprotein-related protein 6 (***LRP6***)	GRK5&6		GRK-mediated phosphorylation of LRP6 activates LRP6 and mediate Wnt/ LRP6 signaling	(Chen et al., 2009)
platelet-derived growth factor receptor-β (***PDGFR***β)	GRK2	HEK293 cells	GRK2-mediated phosphorylation induces desensitization of ***PDGFR***β	(Freedman et al., 2002; Hildreth et al., 2004)
	GRK5	smooth muscle cells	GRK5-mediated phosphorylation induces desensitization of ***PDGFR***β	(Wu et al., 2006)
Other membrane proteins				
epithelial Na+ channel (***ENaC***)	GRK2	cultured salivary duct cells	GRK2-mediated phosphorylation renders ENaC insensitive to inhibition by the ubiquitin ligase Nedd4-2	(Dinudom et al., 2004)
downstream regulatory element antagonist modulator (***DREAM***)	GRK2&6	HEK293 cells	GRK-mediated phosphorylation block DREAM- mediated membrane expression of Kv4.2 potassium channel	(Ruiz-Gomez et al., 2007)
Transcription factors				
Iκ Bα	GRK2&5		GRK-mediated phosphorylation enhances the TNFα-induced NFκB activity	(Patial et al., 2009)
NFκB1 p105	GRK2	Raw264.7 macrophage cells, HEK293 cells	GRK2-mediated phosphorylation of NFκB1 p105 reduces the lipopolysaccharide-induced ERK1/2 activation	(Parameswaran et al., 2006)
receptor-regulated ***Smads***, Smad2&Smad3	GRK2	human hepatocarcinoma cells	GRK2-mediated Smad phosphorylation blocks activin/TGFβ-induced Smad activation, nuclear translocation, and target gene expression	(Ho et al., 2005)
Signaling proteins				

Substrate protein	GRK isoform	Tissue/Cells	Function	References
arrestin2	GRK5		GRK5-mediated phosphorylation prevents activation of Src by 5-HT4 receptor	(Barthet et al., 2009)
Nedd4, Nedd4-2	GRK2	cultured salivary duct cells	unknown; possibly, interference with the binding to epithelial Na+ channel, which is negatively regulated by Nedd4 and Nedd4-2	(Sanchez-Perez et al., 2007)
synucleins (α, β, γ, and synoretin)	GRK2 (α&β isoforms) GRK5 (α isoforms)	COS-1 cells, in vitro	GRK-mediate phosphorylation inhibits synuclein's interaction with phospholipase D2 and phospholipids	(Pronin et al., 2000)
phosducin	GRK2	in vitro	GRK2-mediated phosphorylation of phosducin reduces phosducin's binding to Gβγ	(Ruiz-Gómez et al., 2000)
γ subunit of cyclic nucleotide monophosphate phosphodiesterase type 6 (**PDE**γ)	GRK2	HEK293 cells	GRK2-mediated phosphorylation of PDEα enhances the epidermal growth factor- and trombin-dependent stimulation of ERK1/2	(Wan et al., 2001)
p38	GRK2		GRK2-mediated phosphorylation of p38 at Thr123 impairs p38 activation by MKK6	(Peregrin et al., 2006)
Tumor suppressor gene product adenomatous polyposis coli (**APC**)	GRK2	HEK293	GRK2 interacts with APC via its RGS domain, which results in phosphorylation of a component of the β-catenin destruction complex and inhibition of the canonical Wnt signaling	(Wang et al., 2009)
Na^+/H^+ exchanger regulatory factor (**NHERF**)	GRK6A	HEK293	GRK6A is responsible for constitutive phosphorylation of NHERF at Ser^{289}	(Hall et al., 1999)
Insulin receptor substrate-1 (**IRS-1**)	GRK2	3T3-L1 adipocytes	GRK2-mediated phosphorylation of promotes degradation of IRS-1 leading to reduced insulin signaling	(Usui et al., 2005)
Nuclear proteins				
class II histon deacetylase (***HDAC***)	GRK5	cultured cardiomyocytes, mouse heart	GRK5-mediated phosphorylation of HDAC elevated MEF2-mediated transcription and cardiac hypertrophy	(Martini et al., 2008)
Cytoskeletal proteins				
radixin	GRK2		increased Rac1 activity, membrane protrusion, and cell motility	(Kahsai et al., 2009)

Substrate protein	GRK isoform	Tissue/Cells	Function	References
ezrin	GRK2	HEK293	GRK2-mediated phosphorylation of ezrin links the GPCR activation to the actin cytoskeleton remodeling	(Cant and Pitcher, 2005)
tubulin	GRK2&5	COS-1 cells, in vitro	GRK-mediated phosphorylation of tubulin regulates microtubule assembly and links the GPCR activation to the cytoskeleton remodeling	(Haga et al., 1998; Pitcher et al., 1998; Carman et al., 1998)
Ribosomal proteins				
ribosomal protein P2	GRK2	HEK293	GRK2-mediated phosphorylation of P2 enhances the translational activity	(Freeman et al., 2002)