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Novel Features of G Protein-Coupled Receptor Kinase 4

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Running Title: Novel Features of GRK4

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Abbreviations used: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; βARK, β–adrenergic receptor kinase; HEK, human embryonic kidney; PIP2, phosphatidylinositol bisphosphate

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ABSTRACT

The defining characteristic of G protein-coupled receptor homologous desensitization is that the receptor must be occupied by an agonist or in an activated conformation that mimics an agonist-induced state. In most instances, the mechanistic basis for this characteristic is the high selectivity of G protein-coupled receptor kinases for the activated receptor. Rankin *et al*. (p. XXX) now demonstrate that under at least some conditions the G protein-coupled receptor kinase GRK4 does not display a preference for the agonist-occupied D_1 dopamine receptor. Co-expression of GRK4 and the D_1 receptor in a heterologous system induces phosphorylation of the receptor in the absence of agonist, causing constitutive desensitization and internalization of the receptor. Lacking the normal rapid feedback mechanisms associated with homologous desensitization, a system incorporating constitutively active GRK4 will be prone to dysregulation, perhaps explaining the generally low expression of GRK4. Indeed, considerable evidence suggests that just such dysregulation resulting from mutationally activated GRK4 contributes to the heritable component of human essential hypertension (*Physiol Genomics* **19**:223-246, 2004)

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G protein-coupled receptors (GPCRs) typically respond to agonist stimulation with a time-dependent, rapidly reversible diminution, or desensitization, of the signalling response. Desensitization can be homologous (a receptor desensitized as a result of its own activation) or heterologous (one receptor desensitized as a consequence of the activation of and signalling by a different receptor). The canonical model of homologous desensitization of GPCRs (Van Koppen and Jakobs, 2004) is that the agonist-activated receptor binds and activates a GPCR-selective kinase (GRK) (Benovic *et al.*, 1986), which phosphorylates the receptor on multiple serine/threonine residues. Activation and phosphorylation of the GPCR increases its affinity for arrestin (Benovic *et al.*, 1987). Binding of arrestin to the intracellular loops of the GPCR both sterically hinders the interaction of receptor and G protein and recruits the GPCR into clathrin-coated pits for dynamin-dependent internalization into clathrin-coated vesicles (Goodman, Jr. *et al.*, 1996). The internalized GPCR may be dephosphorylated and rapidly recycled to the plasma membrane (Pippig *et al.*, 1995) or retained in the cell and ultimately degraded, with the choice determined by factors such as the phosphorylation state of other residues (Mason *et al.*, 2002) and the stability of the interaction between the GPCR and arrestin (Oakley *et al.*, 1999; Pan *et al.*, 2003).

This model of homologous desensitization has been validated for countless GPCRs. Still, at almost every step of this process there are examples of exceptions to and deviations from the model. In some cases the deviations represent expanded roles for some of the players, such as GRKs (Fig. 1), in other cases the deviations represent alternative pathways in addition to those in the canonical pathway, and in still other cases it seems that a receptor uses an alternative mechanism instead of the canonical pathway. The GRK can bind to the receptor and influence signalling without phosphorylating the receptor (Perroy *et al.*, 2003; Dhami *et al.*, 2005), GRKs

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phosphorylate other proteins constitutively or upon activation by a GPCR (Pitcher *et al.*, 1998b; Pronin *et al.*, 2000; Hall *et al.*, 1999), arrestin can bind to unphosphorylated receptor (Kim *et al.*, 2004; Chen *et al.*, 2004; Jala *et al.*, 2005), GPCR-activated arrestin can mediate GPCR signalling in addition to internalization (Luttrell and Lefkowitz, 2002; Gurevich and Gurevich, 2003), desensitization and internalization can occur without phosphorylation of the GPCR (Malecz *et al.*, 1998) or without arrestin binding (Pals-Rylaarsdam *et al.*, 1997; Bennett *et al.*, 2001; Bhatnagar *et al.*, 2001; Van Koppen and Jakobs, 2004), GPCRs can internalize via clathrin- and dynamin-independent pathways (Pals-Rylaarsdam *et al.*, 1997; Vickery and von Zastrow, 1999), and dephosphorylation/resensitization can occur in the plasma membrane without receptor internalization (Gardner *et al.*, 2001).

One component of the model that has been constant, and that defines homologous desensitization, has been the strong preference of GRKs for phosphorylation of the agonist-activated receptor over the inactive receptor. The instances when a GPCR is robustly phosphorylated by a GRK without being occupied by an agonist are examples that prove the rule, because the receptor invariably has a high level of inherent or mutationally induced constitutive activity (Ren *et al.*, 1993; Pei *et al.*, 1994; Geras-Raaka *et al.*, 1998; Miller *et al.*, 2003; Marion *et al.*, 2004); the GPCR must be in an active conformation for significant GRK-catalyzed phosphorylation to occur. There is also some evidence that a GRK bound to an activated GPCR might phosphorylate adjacent, inactive GPCRs (Palczewski, 1997), but that does not invalidate the fundamental requirement for an activated receptor to bind and activate the GRK.

In this issue of *Molecular Pharmacology*, Rankin et al. (2006) demonstrate that heterologously expressed dopamine D_1 receptor is constitutively phosphorylated by heterologously expressed GRK4. The D_1 receptor does not have an unusually high level of

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constitutive activity, yet when co-expressed in HEK293 cells with GRK4 it is phosphorylated to such an extent that agonist treatment causes little additional phosphorylation; in this system GRK4 apparently does not distinguish between active and inactive D_1 receptor. GRK4-catalyzed constitutive phosphorylation is associated with reduced dopamine-stimulated cyclic AMP accumulation (desensitization) and receptor internalization. The constitutively phosphorylated residues appear to be close to the C-terminus of the receptor, since truncation at residue Thr404 or combined mutation of Thr428 and Ser431 prevents or substantially decreases constitutive phosphorylation, desensitization, and internalization of the receptor. Thus, the inactive D_1 receptor can be phosphorylated by GRK4, resulting in diminished responsiveness to subsequent stimulation by dopamine.

The seven GRKs are grouped into 3 subfamilies: the retinal subfamily (GRK1/7), the β-adrenergic receptor kinase (βARK) subfamily (GRK2/3), and the GRK4 subfamily (GRK4/5/6). Characteristics of the GRK4 subfamily include predominant localization at the membrane as a result of palmitoylation on C-terminal cysteine residues (for GRK4/6) or interaction between a positively charged domain near the C-terminus and negatively charged membrane phospholipids (GRK5), activation by phosphatidylinositol-bisphosphate (PIP2) binding to an N-terminal domain, and enhanced sensitivity to inhibition by calcium-sensor proteins such as calmodulin (Pronin *et al.*, 1997; Pitcher *et al.*, 1998a; Kohout and Lefkowitz, 2003; Willets *et al.*, 2003). GRK4 is unusual within its subfamily (but similar to GRK1) in that its relatively low sequence homology across species suggests that it is subject to lower evolutionary pressure for sequence conservation and evolving more rapidly than the other members of its subfamily and the βARK subfamily (Premont *et al.*, 1999). Interestingly, GRK1/7 and GRK4 also differ from the other GRKs in tissue distribution. GRK2/3 and GRK5/6 are ubiquitously expressed, whereas GRK1/7

are expressed almost exclusively in the retina, and GRK4 is abundantly expressed only in the testes and expressed at much lower levels in other tissues including the kidney and the brain (Ambrose *et al.*, 1992; Premont *et al.*, 1996; Virlon *et al.*, 1998; Sallese *et al.*, 2000; Willets *et al.*, 2003).

Human GRK4 exists as 4 splice variants, GRK4α, GRK4β, GRK4δ, GRK4γ (Premont *et al.*, 1996). GRK4α is the full-length version, most homologous with the other GRKs. GRK4β is missing the sequence encoded by exon 2, resulting in a 32-residue deletion that encompasses the PIP2 binding domain near the N-terminus. GRK4γ is missing the sequence encoded by exon 15, resulting in a 46-residue deletion near the C-terminus, and GRK4δ, the shortest variant, is missing both alternatively spliced exons. Rankin et al. (2006) determined that the D_1 receptor was constitutively phosphorylated only by co-expression with GRK4α, and not with GRK2, GRK3, or any of the shorter splice variants of GRK4.

This work raises many interesting questions pertaining to the specificity of the response. First, is this a unique characteristic of the D_1 receptor, or will other GPCRs be found to be constitutively phosphorylated by GRK4? GRK4 is capable of phosphorylating and/or desensitizing rhodopsin (Virlon *et al.*, 1998), the follicle stimulating hormone receptor (Lazari *et al.*, 1999), the m2 muscarinic receptor (Tsuga *et al.*, 1998), the luteinizing hormone/chorionic gonadotropin receptor (Premont *et al.*, 1996), and the β_2–adrenoceptor (Premont *et al.*, 1996). GRK4 can also regulate the calcium-sensing receptor (Pi *et al.*, 2005) and is the endogenous GRK that regulates homologous desensitization of two other class C GPCRs in cerebellar neurons, the mGluR1 (Sallese *et al.*, 2000) and $GABA_B$ (Perroy *et al.*, 2003) receptors, in addition to being an endogenous regulator of the D_1 receptor in renal proximal tubule cells (Watanabe *et al.*, 2002; Felder *et al.*, 2002). Interestingly, there is one report that GRK4 causes

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constitutive phosphorylation of the β_2–adrenoceptor in HEK293 cells, with no additional phosphorylation induced by agonist treatment, and also causes enhanced agonist-independent internalization of the receptor (Ménard *et al.*, 1996). It seems likely that GRK4 will be found to catalyze constitutive phosphorylation of additional GPCRs.

A second question is whether constitutive GPCR phosphorylation is restricted to the GRK4 subtype. Rankin *et al*. (2006) determined that the D_1 receptor is not constitutively phosphorylated by GRK2/3, the two members of the βARK subfamily. Although other members of the GRK4 subfamily were not tested in this paper, previous work has shown robust agonist-stimulated phosphorylation of the D_1 receptor by GRK5 in HEK293 cells (Tiberi *et al.*, 1996), suggesting a preference of that kinase for the activated state of the receptor. For the β_2–adrenoceptor, all 3 members of the GRK4 subfamily caused significantly more basal phosphorylation than GRK1-3, but only in the presence of GRK4 was there no additional agonist-induced phosphorylation. A mechanistic basis for the greater propensity of members of the GRK4 subfamily to phosphorylate inactive GPCRs could be their constitutive localization at the membrane, in contrast to GRK2/3 whose translocation to the membrane is aided by free Gβγ produced by GPCR-activated heterotrimeric G proteins. If GRK4 is more likely than other members of that subfamily to exhibit no preference for activated GPCR over inactive receptor, an interesting line of investigation will be to identify the unique features of GRK4 that are responsible for this characteristic.

A system in which a GRK constitutively desensitizes a GPCR seems susceptible to dysregulation, in contrast to the homeostasis conferred by homologous desensitization in which only the activated receptor is desensitized, because an overabundance of the GRK or a mutation that enhances its activity, as in the kidney (see below), could cause perpetual desensitization. Is

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this the reason for the restricted distribution and generally low abundance of GRK4? Is there a unique characteristic of GPCR function in testes that makes it advantageous to have a high level of GRK4 and, hypothetically, constitutive desensitization?

Finally, what is the physiological relevance of the constitutive phosphorylation of the D_1 receptor by GRK4? Significant expression of GRK4 in D_1 receptor-dense brain regions has not been described. In renal proximal tubules, on the other hand, where the D_1 receptor regulates natriuresis, genetic hypertension in rats and human essential hypertension are associated with non-responsiveness to dopamine because of constitutive desensitization of the D_1 receptor (Zeng *et al.*, 2004). GRK4, in particular the GRK4γ splice variant, catalyzes D_1 receptor hyperphosphorylation in renal proximal tubule cells from subjects with essential hypertension (Felder *et al.*, 2002). Essential hypertension is linked to a locus that includes GRK4 (Casari *et al.*, 1995), and is associated with non-synonymous SNPs in the coding region of GRK4 (Speirs *et al.*, 2004). When heterologously expressed with the D_1 receptor in CHO cells, allelic variants of GRK4γ (R65L, A142V, A486V) cause enhanced desensitization and agonist-independent phosphorylation of the receptor, and transgenic mice expressing the A142V variant of GRK4γ, but not wildtype GRK4γ, are hypertensive and lack D_1 agonist-induced diuresis and natriuresis (Felder *et al.*, 2002). The parallels between the work of Rankin *et al*. and the role of GRK4 in the kidney are not exact, because GRK4γ appears to be the variant that regulates the D_1 receptor in renal proximal tubule cells, but in HEK293 cells GRK4γ and its allelic variants, as well as GRK4β and GRK4δ, do not enhance basal phosphorylation of the D_1 receptor or have any effect that can be distinguished from endogenous GRKs {RANKIN2006}. It is possible that the lack of effect of GRK4γ in HEK293 cells can be attributed to differences in the cellular environment, and an interesting line of investigation will be to evaluate the effect of GRK4α, and the effect of

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the SNPs in the context of GRK4α, on D_1 receptor function in renal proximal tubule cells. Despite the discrepancies, the similarity between the observation that GRK4 does not distinguish between inactive and active D_1 receptor in HEK293 cells and the accumulating evidence that GRK4 hyperactivity is the cause of insensitivity to dopamine in essential hypertension suggests that further investigation of the mechanisms of this unusual characteristic of GRK4 will also help to elucidate fundamental mechanisms of the disorder.

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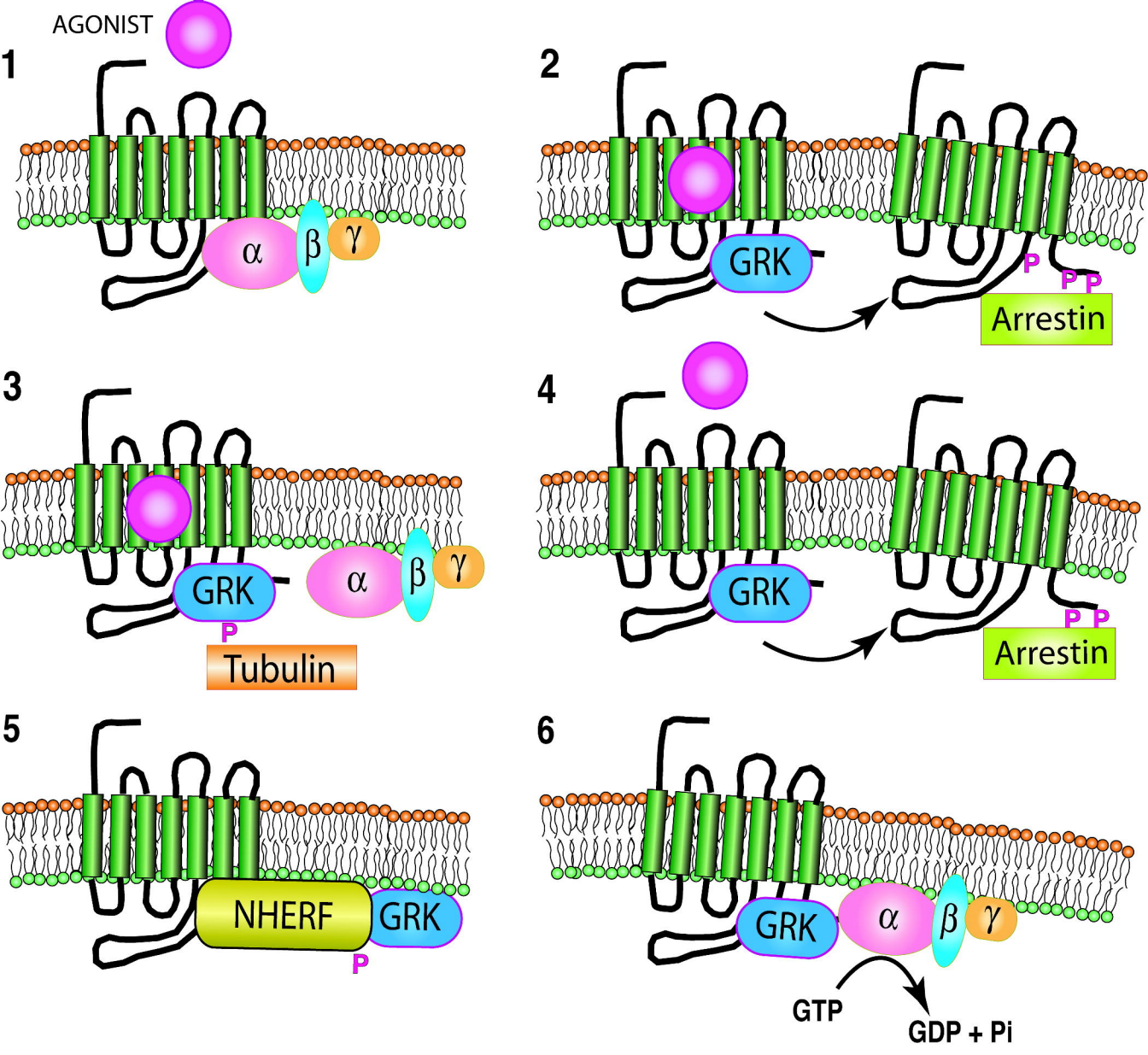
Figure Legend

Fig. 1. Expanded role for G protein-coupled receptor kinases in desensitization and signalling. **1,** A GPCR is shown pre-coupled to a heterotrimeric G protein prior to activation by agonist. **2,** In the canonical model of homologous desensitization, the agonist-activated GPCR binds to and is phosphorylated by GRK, which promotes the binding of arrestin, thus interfering with coupling to the G protein. **3,** GRK binds to the receptor and non-catalytically hinders GPCR coupling to the G protein. Receptor-activated GRK also phosphorylates other proteins such as tubulin. **4,** Rankin *et al*. (2006) demonstrate that GRK4 constitutively phosphorylates a non-agonist-occupied GPCR, leading to desensitization and internalization presumably *via* binding of arrestin. **5,** GRK constitituvely phosphorylates other receptor-interacting proteins such as the Na^+/H^+ exchanger regulatory factor (NHERF). **6,** GRK2/3, in particular, may also decrease signalling by binding to Gα and accelerating its GTPase activity (Willets *et al*., 2003), stimulating the hydrolysis of GTP to GDP and inorganic phosphate.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