cGMP Regulated Protein Kinases (cGK)

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Contents

Abstract cGMP-dependent protein kinases (cGK) are serine/threonine kinases that are widely distributed in eukaryotes. Two genes – *prkg1* and *prkg2* – code for cGKs, namely cGKI and cGKII. In mammals, two isozymes, cGKIα and cGKIβ, are generated from the *prkg1* gene. The cGKI isozymes are prominent in all types of smooth muscle, platelets, and specific neuronal areas such as cerebellar Purkinje cells, hippocampal neurons, and the lateral amygdala. The cGKII prevails in the secretory epithelium of the small intestine, the juxta-glomerular cells, the adrenal cortex, the chondrocytes, and in the nucleus suprachiasmaticus. Both cGKs are major down stream effectors of many, but not all signalling events of the NO/cGMP and the ANP/cGMP pathways. cGKI relaxes smooth muscle tone and prevents platelet aggregation, whereas cGKII inhibits renin secretion, chloride/water secretion in the small intestine, the resetting of the clock during early night, and endochondreal bone growth. cGKs are also modulators of cell growth and many other functions.

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Abbreviations

cGMP-dependent protein kinases, cGK; amino acids, aa; guanylyl cyclase, sGC; soluble guanylyl cyclase, sGC; particulate guanylyl cyclase, pGC; atrio-natriuretic peptide, ANP; brain-natriuretic peptide, BNP; C-type natriuretic peptide, CNP; phosphodiesterases, PDE; cyclic nucleotide-gated, CNG; inositol 1,4,5-trisphosphate receptor I (IP_3RI)-associated cGMP kinase substrate, IRAG; regulator of G protein 2, RGS2; cystic fibrosis transmembrane conductance regulator, CFTR; sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, SERCA; Ca^{2+}-activated maxi-K^+ channel, BK_{Ca}; regulatory myosin phosphatase targeting subunit, MYPT; myosin light chain phosphatase, MLCP; smooth muscle, SM; SM-myosin heavy chain, SM-MHC; SM-α-actin, SMA; nucleus suprachiasmaticus, SCN; circadian time, CT; Na^+/H^+ exchanger 3, NHE3.

1 Introduction

NO is generated by three different isozymes (NO-synthases; NOS 1–3). In many cells, NO increases the concentration of cyclic guanosine monophosphate (cGMP) by activation of the soluble guanylyl cyclase (sGC) (Friebe and Koesling 2003; Katsuki et al. 1977). cGMP is also generated by membrane-bound particulate guanylyl cyclases (pGCs, e.g. GC-A, GC-B, and GC-C). GC-A and GC-B are the major receptors for a family of natriuretic peptides released from the heart and vascular endothelium, like atrio-natriuretic peptide (ANP), brain-natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), whereas GC-C is the receptor for guanylin, an intestinal peptide involved in intestinal fluid regulation (Garbers 1992). Further analysis of the cGMP system identified a number of intracellular targets for cGMP. For example, cGMP binds to cyclic adenosine monophosphate (cAMP)-specific phosphodiesterases (PDEs) and thereby modulates the concentration of cAMP enabling a cross-talk between both cyclic nucleotide pathways (Bender and Beavo 2006; Rybalkin et al. 2003). cGMP and cAMP activate cyclic nucleotide-gated (CNG) cation channels that are an important part of the signal transduction pathway in the visual and olfactory system (Biel et al. 1999; Hofmann et al. 2004). Most cells contain at least one of three cGMP-dependent protein kinases (cGKs): cGKIα, cGKIβ, or cGKII (Feil et al. 2003; Hofmann 2005; Pfeifer et al. 1999) that are targeted by their distinct amino termini to different substrates that are involved in the regulation of different cellular functions.

NO signals not only through the cGMP pathway but has several effects that are independent of the cGMP/cGK signalling pathway (Hess et al. 2005). Interpretation of cGMP effects should include the same precautions, because (1) cGMP has several effectors that may be used simultaneous in various tissues, (2) cGMP might activate directly or indirectly cAMP-dependent protein kinases, and (3) some of the effects of “cGK-specific” activators and inhibitors are not mediated by cGKs (Burkhardt et al. 2000; Daugirdas et al. 1991; Marshall et al. 2004; Wyatt et al. 1991b).

This article will concentrate on some results obtained by total or tissue-specific deletion of the cGK genes and of some of their substrates. We will discuss how cGK signalling might contribute to the maintenance of a "healthy" and physiological status on the one hand and at the same time might affect many aspects that modulate the progression of various diseases. Please note that the physiological function of cGKs are also summarized in the chapters on "cGK Substrates" by J. Schlossmann, "cGMP Kinase-Modulators" by E. Butt, "cGMP kinase as behaviour modifier" by M Sokolowski, "Platelets and blood cells" by U Walter, and "cGMP Signalling in Brain" by R Feil. In addition, several excellent reviews are available for those wanting to delve deep into this topic (Feil and Kleppisch 2008; Feil et al. 2003; Hofmann 2005; Hofmann et al. 2006; Lincoln et al. 2006; Lohmann and Walter 2005; Pfeifer et al. 1999).

2 Properties of cGKs

2.1 Genes, Isozymes, and Structure

cGKs belong to the family of serine/threonine kinases and are present in a variety of eukaryotes ranging from the unicellular organism *Paramecium* to *Homo sapiens* (Francis and Corbin 1999; Pfeifer et al. 1999). Mammals have two cGK genes, *prkg1* and *prkg2* that encode cGKI and cGKII (Orstavik et al. 1997; Sandberg et al. 1989; Wernet et al. 1989). The human *prkg1* gene is located on chromosome 10 at p11.2–q11.2 (52,421,124 bp–53,728,116 bp) and has 15 exons. The N-terminus (the first 90–100 aa) of cGKI is encoded by two alternative exons that produce the isoforms cGKIα and cGKIβ. Their transcripts code for 671 aa (cGKIα) and 686 aa (cGKIβ) yielding proteins with an apparent mass of 76,364 Da (cGKIα) and 77,804 Da (cGKIβ), respectively. The human *prkg2* gene is located on chromosome 4 at q13.1–q 21.1 (82,228,861 bp–82,355,212 bp) and has 19 exons. Its transcript length is 3,431 bp (number of residues 762) yielding a protein with an apparent mass of 87,432 Da.

The enzymes have a rod like structure. They are composed of two functional domains: a regulatory (R) domain and a catalytic (C) domain (Fig. 1). The regulatory domain is further subdivided into the N-terminal domain and the cGMP binding domain containing the high (cGMP I) and low (cGMP II) affinity binding pockets. The two tandem cGMP-binding sites interact allosterically (for details see Francis and Corbin 1999; Pfeifer et al. 1999). Occupation of both binding sites induces a large change in secondary structure (Landgraf et al. 1990) to yield a more elongated molecule (Wall et al. 2003; Zhao et al. 1997). The catalytic domain contains the MgATP- and peptide-binding pockets. Binding of cGMP to both sites in the R-domain releases the inhibition of the catalytic center by the N-terminal autoinhibitory/pseudosubstrate domain and allows the phosphorylation of serine/threonine residues in target proteins. Activation of substrate phosphorylation may be preceded

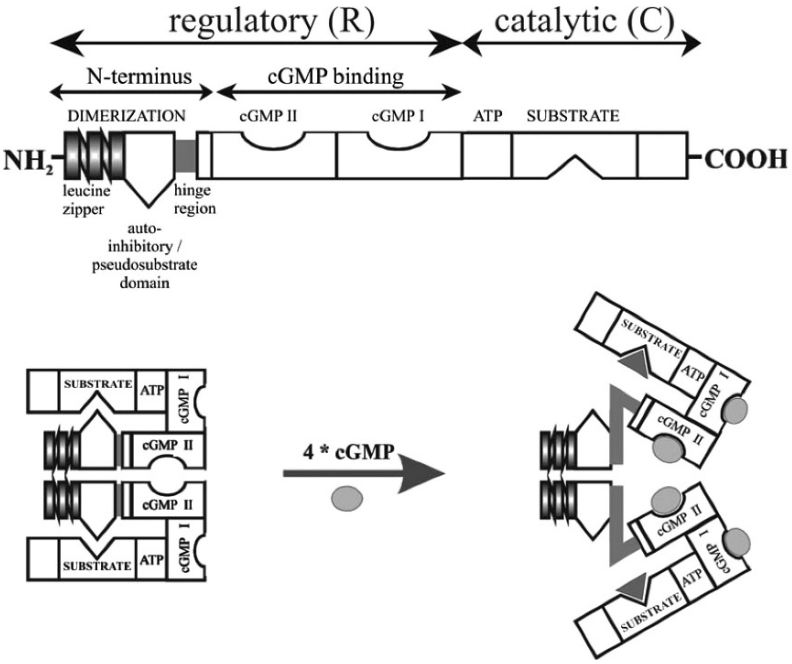

Fig. 1 Structure of cGMP-dependent protein kinase. For details see text

by autophosphorylation of the amino-terminal autophosphorylation site of cGK. Autophosphorylation increases the spontaneous activity of cGKI and cGKII (Francis et al. 2002; Smith et al. 1996; Vaandrager et al. 2003; Wyatt et al. 1991a) and is initiated by the binding of low cGMP concentrations to the high affinity site of cGKI (Hofmann et al. 1985; Smith et al. 2000). In addition to controlling activation and inhibition of the catalytic center, the N-terminus has two other functions: (1) dimerization – cGKs are homodimers that are held together by a leucine zipper present in the N-terminus; (2) targeting – the enzymes are targeted to different subcellular localizations by their N-termini.

The two cGKI isozymes are activated at submicromolar to micromolar concentrations of cGMP with cGKIα being significantly more sensitive than the cGKIβ isoform (Gamm et al. 1995; Ruth et al. 1997). The different activation constants are caused by differences in the leucine zipper, the autoinhibitory/pseudosubstrate domain, and the hinge region. The two cGKI isozymes share 8 cysteines outside of the amino terminus. The cGKIα contains additional cysteines at aa 43 and aa 118. Oxidation of the bovine cGKIα enzyme by Cu^{2+} activates the enzyme through formation of two disulfide bonds between Cys-118 and Cys-196 and Cys-313 and Cys-519 (Landgraf et al. 1991). The biological significance of this activation is not obvious, because oxidation of the enzyme by transition metals is unlikely to occur in vivo. Surprisingly, the cGKIα forms an interprotein disulfide at Cys-43 linking its two subunits in cells exposed to exogenous hydrogen peroxide (Burgoyne et al.

2007). Cys 43 is only present in the Iα isoform. Oxidation of Cys 43 activates the kinase independent of cGMP and has been suggested as an alternative activation mechanism of cGKI in vascular smooth muscle under oxidative stress.

2.2 Tissue Distribution

cGKI is present in high concentrations ($>0.1\,\mu M$) in all smooth muscles, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular endplate, and kidney. Low levels have been identified in cardiac muscle, vascular endothelium, granulocytes, chondrocytes, osteoclasts, and a diverse number of brain nuclei (Feil et al. 2005; Keilbach et al. 1992; Lohmann et al. 1981). The Iα isozyme is found in lung, heart, dorsal root ganglia, and cerebellum. Together with the Iα isozyme, the Iβ isozyme is highly expressed in smooth muscle, including uterus, vessels, intestine, and trachea (Geiselhöringer et al. 2004a; Weber et al. 2007). Platelets, hippocampal neurons, and olfactory bulb neurons contain mainly the Iβ isozyme (Geiselhöringer et al. 2004a). The Iα and Iβ cGKs are soluble enzymes and interact with different proteins through their distinct N-termini. In contrast, cGKII is anchored at the plasma membrane by myristoylation of the N-terminal Gly2 residue. Only the membrane-bound cGKII phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) thereby activating intestinal chloride secretion (Vaandrager et al. 1998). In addition, cGKII is expressed in several brain nuclei, intestinal mucosa, kidney, adrenal cortex, chondrocytes, and lung (de Vente et al. 2001; el-Husseini et al. 1995; Lohmann et al. 1997; Werner et al. 2004).

2.3 Substrates and Signalling Mechanisms

Over ten substrates have been identified that are phosphorylated in vivo by cGKI and several that are modified by cGKII (See Table 1 for details). In addition, a number of proteins exist that are proven substrates in expression system (Table 2). So far, their phosphorylation has not been shown to occur in vivo. The majority of identified substrates modulate the function of other signalling pathways being ion channels, G proteins, and associated regulators or cytoskeletal associated proteins. Details on the function of these proteins will be given in the following sections and in the chapter on "cGK Substrates" and further chapters. The substrate specificity depends on the distinct N-terminus of each isozyme, e.g. the inositol 1,4,5-trisphosphate receptor I (IP_3RI)-associated cGMP kinase substrate (IRAG) interacts only with the N-terminus of the Iβ isozyme (Schlossmann et al. 2000), whereas the myosin phosphatase binding subunit interacts specifically with the N-terminus of the Iα isozyme (Surks et al. 1999). Inhibition of IP_3 synthesis depended specifically on the expression of the Iα isozyme (Ruth et al. 1993; Tang et al. 2003) and could not be achieved by expression of the Iβ isozyme (Meinecke et al. 1994) presumably because only

Table 1 Established cGK substrates and their physiological function(s)

Substrate	Molecular weight (kDa)	cGK isoform	Tissue or cells	Function of phosphorylation	Reference
BK_{Ca}	130	cGKI	SM	Increased open probability; membrane hyperpolarization	(Sausbier et al. 2000)
G-substrate	32	cGKI	Cerebellum	Protein phosphatase inhibitor; initiation of LTD	(Endo et al. 2003)
IP_3 receptor type I	230	cGKI	Cerebellum	Stimulation of calcium release from IP_3 sensitive stores	(Haug et al. 1999; Wagner et al. 2003)
IRAG	125	cGKIβ	SM	Reduced calcium release from IP_3 sensitive stores	(Geiselhöringer et al. 2004b; Schlossmann et al. 2000)
MYPT1	130	cGKIα	SM	Inhibition of myosin phosphatase inhibition by rho kinase; decreased calcium sensitization	(Wooldridge et al. 2004)
PDE5	100	cGKI	SM, Pl	Enhanced cGMP degradation	(Rybalkin et al. 2002)
Phospholamban	6	cGKI	Vascular SM	Enhanced calcium uptake by the Ca-ATPase Serca, faster relaxation?	(Lalli et al. 1999)
RGS2	24	cGKIα	SM	Inhibition of IP_3 generation	(Sun et al. 2005; Tang et al. 2003)
RhoA	22	cGKI	SM, Hippo	Reduced MLC phosphorylation, vesicle trafficking	(Ellerbroek et al. 2003)
Sox9	56	cGKII	Chondrocytes	Bone development	(Chikuda et al. 2004)
Telokin	17	cGKI	SM	Inhibition of MLCK activity	(Walker et al. 2001)
VASP	46/50	cGKI	SM, Pl, Hippo	Regulation of the actin cytoskelet, vesicle trafficking	(Butt et al. 1994; Hauser et al. 1999)
Vimentin	57	cGKI	Neutrophils	Regulation of the cytoskeleton	(Pryzwansky et al. 1995)

Abbreviation: SM, smooth muscle; Pl, platelets; Hippo, hippocampus

the Iα isozyme binds to the regulator of G protein 2 (RGS2) (Tang et al. 2003) and terminates thereby the activity of phospholipase C. The above mentioned interaction of CFTR is specific for the N-terminus of cGKII (Vaandrager et al. 1998). Recent evidence suggested that Ser-293 of VASP, the cGKI-specific site, is also phosphorylated by cGKII in primary renal tubule epithelial cells (Lindsay et al. 2007) indicating that the cGKs might have an overlapping substrate specificity. The cGKI

Table 2 cGK substrates phosphorylated in heterologous systems and with a potential function in vivo

Substrate	Molecular weight (kDa)	cGK Iso-form	Tissue or cells	Potential function of phosphorylation	Reference
Cav1.2 β2a subunit S496	74	cGKIα	HEK cells	Inhibition of L-type calcium current	(Yang et al. 2007)
CFTR	200	cGKII	IEC-CF7cells (Intestinal cell line)	Stimulation of chloride channel	(Vaandrager et al. 1998)
CRP2/CRP4	23	cGKIβ	SM, Neurons	Regulation of smooth muscle tone and smooth muscle specific gene expression; pain perception	(Huber et al. 2000; Schmidtko et al. 2008; Zhang et al. 2007)
Hsp27	27	cGKI	Pl	Decrease of actin polymerisation in vitro	(Butt et al. 2001)
Septin-3	40	cGKI	Brain	Vesicle trafficking	(Xue et al. 2004)
Rap1GAP2	90	cGKI	Pl	Inhibition of Rap1	(Schultess et al. 2005)
Serotonin transporter T276	70	cGK	Brain, CHO-1 cells	Increased serotonin uptake	(Ramamoorthy et al. 2007)
TRPC3	97	cGKI	HEK293	Inhibition of store operated calcium influx	(Kwan et al. 2004)
TxA2 receptor Iα	40	cGKI	HEK293	Desensitization of TP Iα signaling	(Reid and Kinsella 2003)
VASP S239	46/50	cGKII	Primary renal tubule epithelial cells	Loss of lamellipodial protrusion and cell rounding	(Lindsay et al. 2007)
GSK-3β S9	47	cGKII	Chondrocyte/ osteosarcoma	Correct chondrocytes hypertrophy in skeletal bone growth/increase in C/EBPβ binding	(**?** **?**; Zhao et al. 2005)

Abbreviation: SM, smooth muscle; Pl, platelets

isozyme specificity has been tested recently in two transgenic mouse lines expressing only either the cGKIα or the cGKIβ isoform in all smooth muscles (Weber et al. 2007). Both mice lines reconstituted the basic functions of cGKI in several test systems raising the possibility that the substrate specificity is less stringent in vivo than in vitro.

3 cGK Signalling in the Cardiovascular System

3.1 Cardiac Contractility and Remodeling

It is well established that NO and natriuretic peptides (NPs) play an important role in cardiovascular health and disease (D'Souza et al. 2004; Garbers and Dubois 1999; Hofmann et al. 2004; Ignarro 2002; Lloyd-Jones and Bloch 1996; Massion et al. 2003). NO and ANP relax small arteries and arterioles resulting in decreased blood pressure, and NO prevents acute vasoconstriction and thrombosis. In addition, NO/NP signalling modulates cardiac and vascular remodelling processes that are associated with congestive heart failure and atherosclerosis. The molecular mechanisms of cardiovascular NO/NP signalling are not well understood, although cGMP-elevating drugs such as glyceryl trinitrate have been used successfully for the treatment of angina pectoris (Parker and Parker 1998) and the PDE5 inhibitor sildenafil for the treatment of erectile dysfunction and pulmonary hypertension (Lincoln 2004). It is currently accepted that many effects of NO/NP are mediated, at least in part, via cGMP-dependent pathways (Kuhn 2003; Mullershausen et al. 2003; Rybalkin et al. 2003). The major cGK expressed in the cardiovascular system is cGKI (Feil et al. 2003).

The relative importance of cGMP-dependent and cGMP-independent NO effects is controversial (Hare and Stamler 2005; Massion et al. 2003). The combined analysis of conventional and cardiomyocyte-specific cGKI knockout mice demonstrated that cGMP/cGKI contributes to the negative inotropic effect of NO in the juvenile as well as in the adult murine heart (Wegener et al. 2002). However, the NO/cGMP/cGKI pathway does not appear to be involved in the negative inotropic action of acetylcholine (Godecke et al. 2001; Vandecasteele et al. 1999; Wegener et al. 2002). Cardiomyocyte-directed overexpression of cGKIα augmented NO/cGMP inhibition but not muscarinic inhibition of L-type Ca^{2+} channel activity (Schroder et al. 2003). These findings are in line with the results obtained with cGKI-deficient mice and suggest a mechanism for the negative inotropic action of cGKI, namely the inhibition of L-type Ca^{2+} channels. Interestingly, CNP can exert a positive inotropic effect, and this effect was enhanced in the cGKIα overexpressing mice (Wollert et al. 2003). Thus, it appears that NO-stimulated cGMP inhibits, whereas CNP-stimulated cGMP increases, cardiac contractility, and both effects are mediated via cGKI. The dual effects of cGKI on cardiac contractility might be related to distinct cells (e.g. cardiomyocytes versus fibroblsasts), or sub cellular localization of the NO/cGMP/cGKI *versus* CNP/cGMP/cGKI signalling and differences in the amount and duration of cGMP synthesized. We can also not exclude the possibility that yet unidentified alternative signals mediate in concert with cGMP/cGKI the biological effects by either NO or CNP.

The development of cardiac hypertrophy and congestive heart failure is associated with an altered sarcomere organization, fibrosis, and the expression of several fetal genes such as ANP and BNP (Stein and Levin 1998). Mice lacking ANP or the ANP receptor, GC-A, develop pressure-independent cardiac hypertrophy (Holtwick

et al. 2003; John et al. 1995; Kishimoto et al. 2001; Knowles et al. 2001; Oliver et al. 1997). The hypertrophic response of cultured neonatal rat ventricular myocytes to α_1-adrenergic stimulation is suppressed by ANP, NO, or cGMP (Calderone et al. 1998). These results indicate that stimulation of cGMP synthesis by ANP or NO inhibits cardiomyocyte hypertrophy. Whether or not the anti-hypertrophic effect of cGMP is mediated by endogenous cGKI is presently unclear. Adenoviral overexpression of cGKI inhibits myocyte hypertrophy in vitro (Wollert et al. 2002), at least in part via inhibition of the calcineurin-NFAT pathway (Fiedler et al. 2002). However, neither global nor cardiomyocyte-specific ablation of cGKI affected the development of cardiac hypertrophy under basal conditions or in response to pressure overload (Feil et al. 2003). Recently, it was shown that administration of sildenafil suppresses the development of cardiac hypertrophy in response to pressure overload and can even reverse pre-established cardiac enlargement in the mouse (Takimoto et al. 2005). Surprisingly, the potent anti-hypertrophic effect of sildenafil was linked to an apparent decrease in the myocardial cGMP level and an increase in cGKI activity. Thus, the causal relationship between cGMP, cGKI and the anti-hypertrophic action of sildenafil is not clear. It will be interesting to study the effect of sildenafil on pressure-induced hypertrophy in cardiomyocyte-specific cGKI knockout mice.

3.2 Vasorelaxation and Blood Pressure

Mice deficient for cGKI show impaired NO/cGMP-dependent dilations of large and small arteries indicating that the vasorelaxant effects of NO, NPs and other cGMP-elevating agents are mediated, at least in part, via activation of cGKI (Koeppen et al. 2004; Pfeifer et al. 1998; Sausbier et al. 2000; Weber et al. 2007). cGKI inhibits both hormone receptor-triggered (Pfeifer et al. 1998) and depolarization-induced contraction (Geiselhöringer et al. 2004b) by interfering with phospholipase C activation (Tang et al. 2003), by lowering $[Ca^{2+}]_i$ (Geiselhöringer et al. 2004b; Pfeifer et al. 1998), by decreasing Ca^{2+} sensitization of contraction (Bonnevier et al. 2004; Sauzeau et al. 2000; Somlyo and Somlyo 2003), by promoting a reduction of L-type Ca^{2+} current (Weber et al. 2007) and by unknown mechanisms. VSMCs express both cGKIα and cGKIβ (Feil et al. 2002; Geiselhöringer et al. 2004a; Weber et al. 2007; Wolfe et al. 1989). One established in vivo target for cGKIβ is IRAG, which has been identified in a complex with the smooth muscle IP_3 receptor type 1 and cGKIβ (Schlossmann et al. 2000). Phosphorylation of IRAG by cGKIβ inhibits IP_3-induced Ca^{2+} release from intracellular stores in transfected COS cells and in smooth muscle cells (Ammendola et al. 2001; Geiselhöringer et al. 2004b; Schlossmann et al. 2000). Recently, mice have been generated expressing a mutated IRAG protein that does not interact with the IP_3 receptor (Geiselhöringer et al. 2004b). In aortic smooth muscle cells of 9 week-old and older IRAG mutants, cGMP was unable to suppress hormone-induced increases in $[Ca^{2+}]_i$ and contractility indicating that the cGKIβ/IRAG/IP_3 receptor pathway inhibits hormone receptor-triggered intracellular Ca^{2+} release and contraction in vivo (Fig. 2). However, this

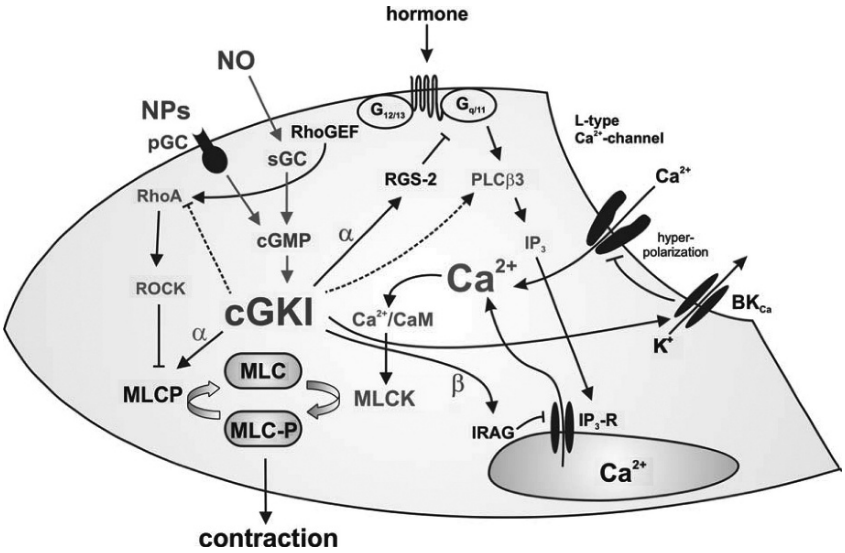

Fig. 2 cGKI Signalling in VSMCs. cGKI-dependent relaxation mechanisms of vascular smooth muscle. Dashed lines, pathways that have not been proven in intact animal tissues. Uninterrupted lines, mechanisms verified in intact animal tissue. Abbreviations are given in the text

pathway is not involved in cGKI-mediated inhibition of contraction initiated by depolarization and activation of voltage-dependent Ca^{2+} channels (Geiselhöringer et al. 2004b).

Additional important cGKI targets that contribute to vasorelaxation (Table 1) have been identified. cGKI activates large-conductance Ca^{2+}-activated maxi-K^+ (BK_{Ca}) channels (Robertson et al. 1993; Sausbier et al. 2000), either by direct phosphorylation (Alioua et al. 1998; Fukao et al. 1999) or indirectly via regulation of a protein phosphatase (White et al. 1993; Zhou et al. 1996). Opening of BK_{Ca} channels results in hyperpolarization of the membrane and closing of voltage-dependent Ca^{2+} channels, thereby, reducing Ca^{2+} influx (Fig. 2). The cGKI may also activate the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), by phosphorylation of the SERCA regulator phospholamban (Koller et al. 2003; Lalli et al. 1999; Raeymaekers et al. 1988). Increased SERCA activity promotes Ca^{2+} re-uptake into the sarcoplasmic/endoplasmic reticulum. The importance of this regulation of the vascular tone is questionable, because deletion of the BK_{Ca} gene (Sausbier et al. 2005) and the phospholamban gene (Lalli et al. 1999) affects the blood pressure only marginally. It has also been suggested that cGKI attenuates early events in hormone receptor-activated pathways including activation of phospholipase C and generation of IP_3 (Hirata et al. 1990; Ruth et al. 1993), perhaps by phosphorylation of receptors (Pfeifer et al. 1998), RGS proteins (Pedram et al. 2000), or phospholipase Cβ (Xia et al. 2001). Reconstitution experiments with cGKI-deficient mice indicated that the inhibition of hormone receptor stimulated Ca^{2+} release can be mediated by the cGKIα and cGKIβ isoforms (Weber et al. 2007). Recently, it has

been shown that the cGKIα isoform binds, phosphorylates, and activates RGS2, which terminates signalling by Gq-coupled receptors for contractile agonists (Tang et al. 2003). These results suggest that, in addition to the cGKIβ/IRAG pathway, the cGKIα/RGS2 pathway may inhibit hormone receptor-triggered Ca^{2+} release and vasoconstriction in vivo (Sun et al. 2005). Another target for cGKI-mediated vasorelaxation is the MLCP (Lee et al. 1997; Surks et al. 1999). The cGKIα isoform interacts with the regulatory myosin phosphatase targeting subunit (MYPT) and activates MLCP (Surks et al. 1999; Wooldridge et al. 2004). Increased MLCP activity would reduce the level of MLC phosphorylation and cause relaxation at constant $[Ca^{2+}]_i$, i.e. Ca^{2+} desensitization of contraction (Fig. 2). Alternative targets of cGKI have been reported (Bonnevier and Arner 2004; Sauzeau et al. 2000; Walker et al. 2001). However, the physiology of the phosphorylation of these proteins remains to be established.

The presented results show that cGKI inhibits receptor-induced vascular smooth muscle contraction by multiple mechanisms including the cGKIβ/IRAG, the cGKIα/RGS2 and the cGKIα/MLCP signalling pathway. The mechanism by which cGKI interferes with depolarization-induced contraction remains controversial. cGKI reduced cardiac Ca^{2+} influx (Mery et al. 1991), most likely by phosphorylation of the calcium channel subunit β2a (Yang et al. 2007) and may contribute thereby to vascular relaxation, because Ca^{2+} influx via the L-type $Ca_v1.2$ calcium channel is essential for sustained contraction of various smooth muscles (Essin et al. 2007; Moosmang et al. 2003; Wegener et al. 2004). The individual contribution of each pathway to cGKI-mediated relaxation presumably varies with the type, the physiological function and the pathology of the vessel.

The effect of cGMP/cGKI signalling on blood pressure is quite complex. Global cGKI-deficiency results in a slight hypertension in young animals, whereas in adult mice the basal blood pressure does not differ between wild type and cGKI-knockout animals (Pfeifer et al. 1998). Furthermore, the smooth muscle specific deletion of the ANP receptor GC-A suggests, that the vascular GC-A is dispensable in the chronic but crucial in the acute regulation of blood pressure by ANP (Kuhn 2005). Interestingly, deletion of eNOS in mice leads to a hypertension in these animals (Huang et al. 1995). Taken together, these studies in SMC GC-A KO and cGKI-deficient mutants suggest that ANP- and NO-dependent, cGMP/cGKI mediated vasorelaxation contributes but is not essential to the regulation of basal, chronic blood pressure. This hypothesis is further supported by recently published results (Michael et al. 2008). These authors inactivated the leucine zipper of cGKIα. The mutated cGKIα interacted poorly with RhoA and showed a higher basal kinase activity than the wild type enzyme. The mutant mice developed within 10 weeks an elevated blood pressure, but had a normal live expectancy. These results are partially contradicted by studies that showed that vascular smooth muscle reconstitution of cGKIα or cGKIβ normalized the elevated blood pressure of $cGKI^{-/-}$ mice (Weber et al. 2007). A potential explanation of the discrepant results could be that active cGKIα that is not localized to its appropriate targets such as RGS2 and MYPT1 phosphorylates proteins not modified in wild type cells and does not phosphorylate its target proteins. These different modified proteins then induce hypertension.

3.3 Vascular Remodeling

In addition to vasodilatation, NO/cGMP signalling has been reported to be involved in the development of vasculo-proliferative disorders, such as restenosis and atherosclerosis. The analysis of transgenic mice showed that NO can both promote (Chyu et al. 1999; Detmers et al. 2000; Kuhlencordt et al. 2001a; Ozaki et al. 2002; Sennlaub et al. 2001; Shi et al. 2002; Tolbert et al. 2001) and inhibit (Chen et al. 2001; Knowles et al. 2000; Koglin et al. 1998; Kuhlencordt et al. 2001b; Moroi et al. 1998; Rudic et al. 1998) pathological vascular remodelling. These findings might explain why NO-generating drugs have not been reported to limit the progression of atherosclerosis or restenosis in humans (Lablanche et al. 1997). The opposing effects of NO on vascular remodelling might depend on the spatiotemporal profile of its production (cellular source, time and quantity) and are probably mediated by different cellular and molecular mechanisms (Poon et al. 2003).

A key process in vascular remodelling is the phenotypic modulation of VSMCs from contractile to synthetic phenotype (Berk 2001; Dzau et al. 2002; Owens et al. 2004; Schwartz et al. 1995). High concentrations of NO inhibit VSMC growth in vitro, even in the absence of cGKI (Hofmann et al. 2006), whereas the "growth-promoting" effect of cGMP was absent in primary VSMCs isolated from cGKI-deficient mice (Weinmeister et al. 2008). Recent evidence shows that the "growth-promoting" effect of cGKI in primary smooth muscle cells is caused by an increased adhesion of the wild type VSMCs to the substrate during the establishment of the cell culture (Weinmeister et al. 2008). These investigations demonstrated also that cGMP slightly inhibited the growth of cGKI-positive but not cGKI-deficient VSMCs, if the cells were passaged several times (Fig. 3).

Most likely, the cGKI-dependent regulation of "cell growth" is mediated by affecting gene expression (Eigenthaler et al. 1999; Lincoln et al. 2001; Pilz and Casteel 2003). VSMCs can reversibly change their phenotype from a differentiated, "contractile" phenotype with high levels of smooth muscle (SM)-specific gene expression to a de-differentiated, "synthetic" phenotype with reduced levels of SM-specific gene expression (Lincoln et al. 2006; Owens et al. 2004). This phenotypic switching plays an important role in the development of vascular diseases: in acutely injured blood vessels, e.g. after balloon angioplasty, VSMCs proliferate and migrate from the medial layer of the vessel wall contributing to a "neo-intimal" layer, and the majority of SM-like cells found in atherosclerotic plaques appear to represent de-differentiated VSMCs originating from the medial layer (Lincoln et al. 2006; Owens et al. 2004). The regulation of VSMC phenotypic switching is complex and mediated by multiple factors, but it is clear that de-differentiated VSMCs are a major cell type responsible for the generation of vascular lesions (Kawai-Kowase and Owens 2007). Primary VSMCs cultured in vitro undergo changes similar to those observed in neo-intimal smooth muscle-like cells, including phenotypic de-differentiation, decreased expression of SM-specific genes, and loss of cGKI (Boerth et al. 1997; Cornwell et al. 1994; Lincoln et al. 2006). When these de-differentiated, cGKI-deficient VSMCs are transfected with expression vectors encoding cGKI to restore physiologic levels of cGKI activity, the cells develop a more contractile phenotype,

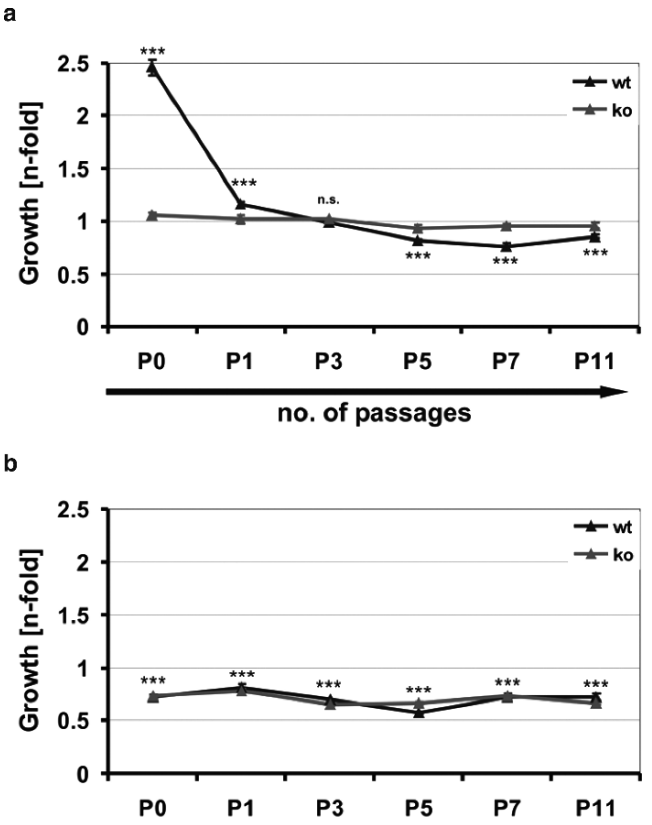

Fig. 3 Growth properties of cultured VSMCs. Growth performance of primary (P0) up to passage 11 (P11) VSMCs in response to 8-Br-cGMP and 8-Br-cAMP. Cell number was measured by the tetrazolium salt MTS (MTS assay). **a** Cells were treated with 0.1 mM 8-Br-cGMP, or **b** 0.1 mM 8-Br-cAMP, respectively. Growth was normalized to control (untreated cells) (>1 = increased growth, 1 = no growth, <1 = decreased growth in comparison to control). **a** Growth is significantly increased in primary wt cells in response to 8-Br-cGMP ($^{***}, p < 0.001$), whereas growth is significantly reduced in subcultured ($>$P5) cells. Treating ko cells with 8-Br-cGMP has no effect on growth. **b** 8-Br-cAMP significantly inhibits growth in wt and ko cells. n = 5–8 wells. Error bars represent SEM

increase expression of SM-specific genes such as SM-myosin heavy chain (SM-MHC), SM-α-actin (SMA), and calponin, and reduce production of extracellular matrix proteins and growth-related genes. These results suggest that cGKI may contribute to the switch from synthetic/proliferative to contractile VSMCs.

Most SM-specific promoters, including the SM-MHC, SMA, and calponin promoter, contain multiple CArG (CC(AT)6GG) elements recognized by the ubiquitously expressed serum response factor (SRF) (Liu and Olson 2006). Expression of these genes depends on the interaction of SRF with multiple cofactors, including the cysteine-rich LIMonly proteins CRP1 and CRP2/smLIM (smooth muscle LIM protein) (Liu and Olson 2006). Recently, a new member of the CRP family was

identified through a yeast two hybrid screen that used cGKIβ as bait (Huber et al. 2000). This protein was independently cloned from a rat brain and human intestinal cDNA library (Karim et al. 1996; Okano et al. 1993) and was named "CRP2" and recently been renamed to "CRP4" (Zhang et al. 2007). CRP4 is phosphorylated by cGKI in vitro and in vivo (Schmidtko et al. 2008; Zhang et al. 2007). cGKI was required for maintaining SM-specific gene expression in several differentiated smooth muscle cell lines, and enhanced SRF- and GATA6-induced differentiation of pluripotent embryonal cells into smooth muscle cells (Zhang et al. 2007). CRP4 was associated with SM-specific promoters and mediated positive transcriptional effects of cGKI on SM-specific gene expression. These results establish for the first time a plausible link for the effect of cGKI on gene expression.

The above discussed results led to the suggestion that cGMP/cGKI signalling might also contribute to the phenotypic modulation and growth of VSMCs during pathological vascular remodelling in vivo (Sinnaeve et al. 2002; Zhang et al. 2007). To test this hypothesis, the consequences of postnatal smooth muscle-specific cGKI deletion were studied in wild type and ApoE-deficient mice. Smooth muscle-specific deletion of cGKI did not affect restenosis in response to carotid ligation in normo-lipidemic wild type mice nor in apoE-deficient mice (Lukowski et al. 2008). Continuous elevation of cGMP by sildenafil had no effect on neointima proliferation after carotid ligation. Furthermore, VSMC-specific deletion of cGKI had no effect on vascular remodelling after wire induced removal of the endothelial cell layer. These studies are consistent with the conclusion that the role of the smooth muscle cGMP/cGKI pathway is negligible in vascular remodelling in vivo under normo-lipidemic condition. An identical conclusion has been published by Sinnaeve and coauthors (Sinnaeve et al. 2002). Gene transfer of the wild type cGKIβ isoform had no effect on restenosis in vivo. These results contrast those reported above and suggest that cGMP/cGKI signalling is not critically involved in vascular restenosis in vivo. Further experiments with different cGKI-deficient mouse models and cultured VSMCs are required to decipher the exact effect of cGMP/cGKI on vascular smooth muscle differentiation/dedifferentiation.

4 cGKII

cGK type II was originally identified and cloned from intestinal specimens (Lohmann et al. 1997). Deletion of the *prkg2* gene revealed a number of interesting phenotypes including behaviour and clock effects, intestinal water secretion, bone growth and renal effects.

4.1 Neuronal Effects of cGKII

cGKII, a signal transducer of NO/cGMP, is widely distributed throughout the mammalian brain including regions thought to be related to emotional behaviour, e.g. cortex, hippocampus, the amygdala and midbrain raphe nuclei (el-Husseini et al.

1995; Werner et al. 2004). cGKII is involved in the control of anxiety-like behaviour and the behavioural effects of ethanol (Werner et al. 2004). Wild type and cGKII deficient mice showed marked differences in their ethanol consumption in a free choice-test without a general difference in place preference. When given free access to two bottles filled with water and ethanol, respectively, cGKII$^{-/-}$ mice consumed significantly more ethanol over the course of 6 days, and this effect, again, was observed for two different genetic backgrounds.

Many behavioural responses of animals (e.g. feeding, drinking and locomotor activity) and the underlying neuro-humoral activities are organized in circadian rhythms. Both cGKI and cGKII have been reported to be expressed in subpopulations of SCN neurons (el-Husseini et al. 1995; Oster et al. 2003; Revermann et al. 2002). Mice with an inactivated cGKII gene displayed a virtually normal spontaneous circadian rhythm and unaltered expression patterns of the clock genes *mPer1* and *mPer2* supporting the view that the circadian clock is still intact in the absence of cGKII (Oster et al. 2003). Disruption of the cGKII gene, however, resulted in an impaired light-induced entrainment of the circadian clock within a definite time window. Compared to wild type mice, the phase delay of the clock induced by a light pulse applied at circadian time (CT) corresponding to early night, CT14, was reduced by nearly 50%. The phase advance of the clock induced by a light pulse applied at CT22, corresponding to late night, was not affected in cGKII knockout mice (Oster et al. 2003). At the molecular level, cGKII knockout mice displayed marked differences to wild type mice in light induction of two clock genes during the early period of the night: induction of *mPer1* was enhanced and induction of *mPer2* was strongly reduced. The absence of cGKII did not affect light induction of these genes during late night. In contrast to the results obtained with cGKII-deficient mice, pharmacological studies have placed the cGKs into the signalling pathway for phase advances (Mathur et al. 1996; Weber et al. 1995), and a recent in vitro study reported an essential role of cGKII in the progression of the circadian cycle (Tischkau et al. 2004). The reason for these discrepancies are not clear, but may be explained in part by effects of inhibitors not related to cGKII-inhibition as well as by differences in the experimental systems used, e.g. slice cultures versus whole animals. Furthermore, a function of cGKI, which is also expressed in the SCN (Revermann et al. 2002), cannot be excluded. Conditional knockout mice lacking cGKI in the SCN did not reveal a significant effect on circadian rhythmicity.

4.2 Peripheral Effects of cGKII

4.2.1 Intestine

cGKII is an important regulator of gastrointestinal secretion (Pfeifer et al. 1996). cGKII is located in the secretory epithelium of the small intestine and stimulates chloride and water secretion possibly through phosphorylation of CFTR (Vaandrager et al. 1998). cGKII increased Na^+-absorption in the small intestine

by inhibition of the Na^+/H^+ exchanger 3 (NHE3) through interaction with the G-kinase anchoring protein NHERF2 (Cha et al. 2005; Vaandrager et al. 2000). Stimulation of the cGMP signalling cascade by toxins causes diarrhoea as the *Escherichia coli* heat stable toxin (STa) and guanylin activate the GC-C and thereby increase water secretion in the small intestine. Correspondingly, STa- did not induce diarrhoea in cGKII knockout mice (Pfeifer et al. 1996).

4.2.2 Kidney

cGMP affects blood pressure not only by directly regulating the vascular smooth muscle tone but also by regulation of the renin and aldosterone secretion (Kurtz et al. 1998; MacFarland et al. 1991; Nikolaev et al. 2005). Renin secretion is enhanced by NO through cGMP-dependent inhibition of cAMP hydrolysis mediated by PDE 3 (Kurtz et al. 1998). In contrast, ACTH-dependent aldosterone secretion is inhibited by ANP through cGMP-dependent stimulation of cAMP hydrolysis mediated by PDE2 (MacFarland et al. 1991; Nikolaev et al. 2005). In addition, it was reported that cGMP analogs reduced renin secretion from isolated kidney or juxtaglomerular cells (Henrich et al. 1988; Schricker and Kurtz 1993). Kidney expresses both isozymes of cGK. cGKII is localized together with storage granules in juxtaglomerular cells (Gambaryan et al. 1996). Renin secretion from juxtaglomerular cells was increased in cGKII-deficient mice (Wagner et al. 1998) suggesting that cGMP has a dual role in the regulation of renin secretion. cGKII has been detected in rat and murine zona glomerulosa cells of the adrenal gland (Gambaryan et al. 2003) and might regulate aldosterone secretion. The overexpression of cGKII in rat zona glomerulosa cells enhanced the production of aldosterone (Gambaryan et al. 2003). Likewise, a low salt diet activating the aldosterone system enhanced expression of cGKII (Gambaryan et al. 2003). In contrast, cGKII deletion did not alter basal or low salt stimulated aldosterone secretion in mice. So far cGKII deletion has not affected resting blood pressure. Therefore, it is unlikely that the above reported findings are relevant for the overall blood pressure regulation in resting mice and other animals.

4.2.3 Bone Growth

The endochondral ossification of bones is stimulated by the C-type natriuretic peptide (CNP) (Chusho et al. 2001). Overexpression of CNP rescued achondroplasia, which was induced by a defect in fibroblast growth factor receptor 3 signalling (Yasoda et al. 2004). cGKII knockout mice are dwarfs, which develop short bones caused by a defect in the endochondral ossification at the endochondral plate (Pfeifer et al. 1996; Talts et al. 1998). This defect was not rescued by CNP overexpression indicating that cGKII is essential for endochondral bone development (Miyazawa et al. 2002). Interestingly, the regulation of body size was also impaired in the cGMP kinase deficient nematode *egl4* (Nakano et al. 2004). Furthermore, cGKII-deficient rats exhibited an expanded growth plate and impaired bone healing. The

observed accumulation of postmitotic but nonhypertrophic cells in these rats might be caused by induction of the transcription factor Sox9 and decreased phosporylation of GSK3β leading to hypertrophic differentiation of chondrocytes (Chikuda et al. 2004; Kawasaki et al. 2008; Zhao et al. 2005).

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