TRACKING FUNCTIONS OF cGMP-DEPENDENT PROTEIN KINASES (cGK)

Suzanne M. Lohmann and Ulrich Walter

Institute of Clinical Biochemistry and Pathobiochemistry, University of Wuerzburg Medical Clinic, Josef Schneider Str. 2, 97080 Wuerzburg, Germany

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1. ABSTRACT

The options available for distinguishing the effects of cGMP mediated by cGK versus those mediated by other cGMP targets are discussed and evaluated. These include the unnecessary but often sole reliance on synthetic, small-molecule activators and inhibitors of cGK which are increasingly recognized as deficient in specificity. Other important adjunct options include cGK overexpression using adenoviral vectors and transgenic animals, or use of cGK-deficient systems, i.e. cells which have spontaneously lost cGK during repetitive passaging in cell culture, cells treated with siRNA, or genetically-engineered cGK-deficient mice. Finally, cGK-dependent phosphorylation of substrates such as vasodilator stimulated phosphoprotein (VASP) and phosphodiesterase 5 (PDE 5) is described as a useful monitor of cGK presence and activity associated with physiological functions or dysfunctions of signaling pathways.

2. INTRODUCTION

The allocation of cGMP-dependent cellular functions to mammalian cGMP-dependent protein kinase (cGK) ideally requires analyses that distinguish activation of specific isoforms of cGK (I-alpha, I-beta, and II) from activation of other cGMP targets such as cGMP-regulated channels and PDEs, as well as from potential cGMP crossactivation of cAMP-dependent protein kinase. The quest of identifying cGK functions has been propelled by an evolution of methods with ever-renewing promise of greater specificity, a promise which gradually dissipates over time as a new set of caveats and cautions are recognized. A highly trustworthy evaluation of cGK functions requires careful weighing of many diverse pieces of evidence. Therefore, here, a spectrum of methods and insights which have have contributed to our understanding of cGK functions will be discussed. Recognizing inherent weaknesses in any single method, we however believe that the sum of results from diverse technologies can reach a consensus concerning certain cGK functions. Since cGK functions in the cardiovascular system have been recently extensively reviewed (1, 2), and additional cGK functions will be examined in this volume by other authors, our review will focus primarily on the methodology and criteria used for identification of cGK functions.

3. AGENTS REGULATING cGK I AND cGK II ACTIVITY

3.1. Physiological agents

A wide range of hormones, neurotransmitters, drugs, and toxins are capable of increasing intracellular cGMP via two major signaling pathways involving natriuretic peptides (NPs), guanylins, and nitric oxide (NO) (3, 4). Since cGMP has many targets, as mentioned above, investigations have attempted to decipher which target(s) are involved in mediating individual functions elicited by physiological agents. Definition of these signaling pathways is important in order to identify specific diagnostic and therapeutic sites of intervention. An early approach was to utilize membrane-permeant cGMP analogs with some degree of specificity/selectivity for the various cGMP targets. The limitations discussed below emphasize

the necessity of supplementing this approach, whenever possible, with others.

3.2. Synthetic mediators and inhibitors
3.2.1. cGMP analogs and protein kinase inhibitors
cGMP analogs, and a few other agents, which either activate or inhibit cGK and additional cGMP targets are shown in Table 1 (5-35). Data from a number of sources is shown in order to demonstrate the possible range of values depending on assay conditions and other variables.

Close examination of the parameters shown quickly convinces that a given concentration of these agents can interact with multiple targets. Thus, optimally, a number of carefully selected analogs/agents need to be utilized to narrow down a cGMP effect, if possible, to the relevant target. This process is however compromised by incomplete information on many analogs, in particular with regard to their effects on PDEs and any number of cGMP-regulated channels. cGMP stimulation or inhibition of PDEs can alter cAMP levels and PKA-dependent functions. Regulatory, non-catalytic, so-called GAF, domains are present in PDE 2 (previously called cGMP-stimulated PDE, cGS-PDE), PDE 5 (cGMP binding, cGMP-specific PDE, cGB-PDE), and PDE 6 (36). cGMP competes with cAMP at the catalytic site to inhibit PDE 3 (previously called cGI-PDE) (36), at cGMP concentrations similar to those that activate cGK (8, 37, 38), and thus increase cAMP. In contrast to the native cGMP increased by physiological activators, membrane-permeable cGMP analogs inhibit PDE 3 with different efficiences (8), such that 100 μM 8-Br-cGMP apparently reaches intracellular concentrations sufficient to inhibit PDE 3 in atrial myocytes (38), whereas 100 μM 8-pCPT-cGMP does not in platelets or perhaps vascular smooth muscle cells (37, 39). Nevertheless, 1 mM 8-Br-cGMP inhibition of aortic ring contraction and Ca^{2+} transients seemed to be abolished in cGK I deficient mice (40).

Note that the channel data shown in Table 1 has been obtained nearly exclusively with the photoreceptor type channel, whereas there are data indicating that some Rp-isomer analogs can have opposite effects on other cGMP-regulated channels such as the olfactory type channel (26). The photoreceptor rod-type cyclic nucleotide-gated cation channel has been shown to be expressed in vascular endothelium and smooth muscle (41), however, information on the extent of expression of these and other types of such channels in many tissues is lacking. Recently, aquaporin-1 was reported to be a cGMP binding and cGMP-gated ion channel (42). cGMP and cGMP analogs which activate (8-Br-cGMP, Sp-8-Br-cGMPS) or inhibit (Rp-8-pCPT-cGMPS) cGK appear to have minimal effect on the Rap guanine nucleotide exchange factor Epac which has recently been described as a new target of cAMP action (25). See the beginning of section 4 for comments on cGK inhibitors, and section 4.1 for a discussion of possible direct activation of PKA by cGMP and its analogs.

Furthermore, the data for protein kinases shown in Table 1 is limited to purified kinases in *in vitro* assays.

In vitro, the relative potency order for stimulatory cGMP analogs varies with respect to different cGKs, and indictes some preference of cGK I isoforms for PET-analogs, in contrast to a cGK II preference for 8CPT-analogs.

cGK I-alpha: 8-Br / 8-Br-PET / PET > 8CPT
cGK I-beta: 8-Br-PET > PET > 8-Br / 8CPT
cGK II: 8CPT > 8-Br > PET

This trend is observed to some extent for inhibitory cGMP analogs as well.

cGK I-alpha: Rp-8-Br-PET > Rp-8CPT
cGK I-beta: "   "
cGK II: Rp-8CPT > Rp-8-Br-PET

However, the concentrations of cGMP analogs which effectively activate or inhibit the kinases *in vitro* cannot be straightforwardly applied to studies with intact cells. Each analog has its own relative lipophilicity (5) that defines its membrane permeating properties, plus has its own susceptability to hydrolysis by PDEs (8, 17), such that the concentration an analog reaches at intracellular sites, as well as whether that concentration corresponds to cGMP levels elicited by physiological agents, is largely unknown. Immediate actions of cGMP analogs which normally require preincubation for cell permeation is an indication for unspecific or extracellular effects, not for activation of intracellular targets like cGK. Thus, for several reasons, the number of targets that extracellularly applied agents might eventually activate or inhibit is uncertain. Careful use of a palette of analogs to determine the inclusion and exclusion of targets is recommended, but adjunct use of additional methodologies discussed below is highly important. When properly used, these methodologies have added a greater level of certainty to the assignment of cGMP effects to targets like cGK.

3.2.2. Adenoviral cGK vectors
cGK I-alpha, I-beta, and cGK II cDNA can be introduced into easily-transfected cells using plasmids, but more efficiently into transfection-resistant cell lines and primary cells using adenoviral vectors (43-46). This direct expression of cGK tests whether exogenous cGK can mimic effects of a physiological agent which raises endogenous levels of cGMP. Pitfalls include that any excessive cGK expression could theoretically evoke an effect which physiological cGK levels do not. In general, however, the cGK levels achieved are not excessively high and can be determined by the number of infecting adenovirus used (44, 47-49). Also, adenoviruses themselves could have non-specific effects on certain functions, however, control adenoviral vectors containing catalytically inactive (Lys-Ala) cGK mutants have been used to rule out such effects (cGK Iβ-K405A (47, 50); cGK Iα-K390A and cGK II-K482A, A. Smolenski and S.M. Lohmann unpublished). An additional complexity is that the observed effect of a physiological agent can reflect the integration of opposing effects of more than one target of cGMP. This is the case for atrial natriuretic peptide (ANP) inhibition of adrenocorticotrophic hormone (ACTH) / cAMP - stimulated aldosterone release. The inhibition caused

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Table 1. Activators and inhibitors of cGK and other targets of cGMP

Analog	cGK I-alpha	cGK I-beta	cGK II	PKA type I	PKA type II	PDE 2	PDE 3	Rod photo-receptor channel	Relative[5] lipophilicity
			Ka (μM)				Ki (μM) (IC50 if shown)	EC50 (IC50 if shown)	
Activators									
cGMP	0.11[6,7,10,23]	0.5[6]	0.04[6]		60[7,8]	0.35[8]	0.14[8]	40[9,18]	1[1]
		0.25[10]			68[11]			17[12]	
	0.092[13]		0.8[13]					24[14]	
	0.047[15]	0.26[15]			4.7[15]				
	0.1[16]	1.27[16]							
8-Br-cGMP[3] (low hydrolysis[8,17])	0.01[6]	1.0[6]	0.025[6]	2.8[17]	12[8,17]	1250[8]	8[8]	1.6[12]	2.5[1]
	0.026[10,17]	0.21[10]						6.7[18]	
	0.018[15]	0.071[15]			6.3[15]				
	0.3[16]	12[16]							
8-pCPT-cGMP (~no hydrolysis[8,17])	0.04[6,11]	0.9[6]	0.0035[6]	0.74[17]	1.3[17]	0[8]	385[8]	0.5[18]	56[1]
	0.05[10,17]	0.44[10]	see also[21]		7[8,11]				
8-APT-cGMP	0.007[10]	1.37[10]							
PET-cGMP	0.03[6]	0.05[6]	0.06[6]						50[1]
	0.026[10,17]	0.02[10]	see also[21]						
	0.016[13]		4.7[13]						
	0.027[17]			16.5[17]	8.7[17]				
8-Br-PET-cGMP	0.013[10]	0.009[10]	see also[21]					64 IC50[9]	115[1]
								(agonist at high concentration)	
Sp-8-Br-PET-cGMPS	2.6[22]	2.5[22]			> 1000[22]			105 IC50[9]	182[1]
cAMP	39[6,7,8]	18[6]	12[6]		0.08[7,8]			1500[14]	1[2]
	5[15]	2[15]			0.044[15]				
	13[13]		83[13]						
8-Br-cAMP (moderate hydrolysis[7,17])	5.8[6,7,17]	10[6]	6[6]	0.065[17]	0.05[7,17]				1.8[2]
	0.72[15]	0.52[15]			0.007[15]				
8-pCPT-cAMP (moderate hydrolysis[7,17])	0.11[7]				0.05[7]		25 IC50[24]		36[2]
	0.2[17]			0.007[17]	0.009[17]				
Sp-5,6-DCl-cBIMPS (very low hydrolysis[7])	10[6,7]	27[6]			0.03[7]				79[2]
				Ki (μM) (IC50 where shown)				IC50 (EC50 where shown)	
Inhibitors									
cGMP analog inhibitors									
Rp-cGMPS	20[11]				20[11]			1200 EC50[12]	1.3[1]
								(antagonist for olfactory)[26]	
Rp-8-Br-cGMPS	3.7[15]	1.8[15]			25[15]			173 EC50[18]	3.3[1]
Rp-8-pCPT-cGMPS[31]	0.5[6,11]	0.6[6]	0.5[6]		8.3[11]			poor agonist[18,26]	68[1]
	18.3 IC50[13]		0.16 IC50[13]					(antagonist at high conc.)[18,26]	
	see also[35]		see also[21]						
Rp-8-Br-PET-cGMPS[31]	0.035[22]	0.03[22]	see also[21]		11[22]			25 IC50[9]	115[1]
	see also[35]								
cAMP analog inhibitors									
Rp-cAMPS	53[6]	14[6]	50[6]					435 IC50[26]	1.3[2]
Rp-8-Br-cAMPS	3.8[15]	3.5[15]			weak[15]				2.4[2]
Other inhibitors									
H89[29,30,31]	0.5[27]				0.05[27]				
KT 5823[28,31,32]	0.23[27]				>10[27]				
	see also[35]								
PKI[107]	(- - - - - - - - - - - - - - varies for peptide length, amidation, etc.[30,33] - - - - - - - - - - - - - -)								
DT-2	0.012[34]				16.5[34]				
	see also[35]								
DT-3	0.025[34]				493[34]				

[1,2] lipophilicity is expressed relative to that of [1]cGMP (1) or [2]cAMP (1), respectively, [3] Abbreviations: Br: bromo; pCPT: para-chlorophenylthio; APT: 8- (2-aminophenylthio); PET: beta-phenyl-1, N^2-etheno; Sp-5,6-DCl-cBIMPS: 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole-3', 5'-monophosphorothioate, Sp-isomer; Rp: Rp-diasteromer, [21]Data not shown since it examines effects on phosphorylation in rat intestinal brush border vesicles, instead of effects on purifed protein kinases, [35]Data not shown since it reports the IC50 for inhibiting vasodilation in rat cerebral arteries, instead of effects on purified protein kinases.

by ANP is mediated by cGMP stimulation of PDE 2 catalytic activity to lower cAMP levels (51). In contrast, cGMP activation of cGK II stimulated basal aldosterone release (49). In another case, cGMP inhibition of PDE 3 caused elevation of cAMP and stimulation of renin release (52), whereas cGMP activation of cGK II inhibited renin release (48), an effect absent in cGK II null mice (53). Indeed, dual effects of NO which cause both stimulation and inhibition of renin release have been observed (48, 52, 53).

3.2.3. Constitutive cGK mutants

Even expressed cGK still has to be activated by cGMP which can then act on other targets. However, since the expression of exogenous cGK (but not that of other targets) is being increased, any observed functional changes must, most likely, be ultimately traced to cGK. Alternatively, various types of constitutive cGK which are active without added cGMP can be expressed. The different types of mutants exploit special features of cGK structure/function. cGKs consist of dimers of two identical monomers, each of which contain an N-terminal regulatory domain and a C-terminal catalytic domain. An autoinhibitory region in the regulatory domain contains a pseudosubstrate sequence that is responsible in part for autoinhibition of the catalytic domain. This autoinhibitory interaction is disrupted by a cGMP-induced conformational change in cGK. These domains have been reviewed many times (54-56). Cell expression studies have been carried out with constitutively active cGK obtained either by N-terminal truncation (delta-1-92 amino acids) (57) of the so-called dimerization domain of cGK I which includes the autoinhibitory region, or by deletion of the entire regulatory domain (~ 340 amino acids) of cGK I to retain only the catalytic domain (58-61), or by introduction of mutations which interfere with the electrostatic interaction between the positively charged residues of the pseudosubstrate autoinhibitory region and the negatively charged residues of the catalytic domain cleft, and have other steric effects (62, 63). The latter has been done by mimicking autophosphorylation in the autoinhibitory region of cGK I-alpha (Ser64Asp or Thr58Glu), cGK I-beta (Ser79Asp), and cGK II (Ser126Glu) (62, 64-66), or by mutation of basic residues (Arg^{118}, Arg^{119}, Val^{125}) to Ala in the pseudosubstrate autoinhibitory region to produce a constitutive cGK II (63).

Some caveats in the expression of constitutively active mutants should be considered. The amount of activity achieved with these mutants in intact cells depends at least partly on their stability; e.g. truncation of the first 103 amino acids of cGK I-beta, rather than after the first 92 (57), led to greatly enhanced cGK I degradation (A. Smolenski and S.M. Lohmann, unpublished data). Another potential, little analyzed, caveat in the expression of constitutive cGK is that alteration of cGK by point mutations, or particularly by large truncations, may have effects on cGK binding to other proteins which anchor it to intracellular sites and thus possibly restrict its activity to specific substrates. cGK I-alpha and cGK I-beta isoforms differ in their first ~100 amino acids and, except for troponin T which binds both isoforms (67), some anchor proteins bind either Iα (myosin binding protein of the myosin light chain phosphatase, the G-kinase anchor protein GKAP42, and vimentin (latter however not tested with cGK I-beta) (68-70), or I-beta (the IP_3 receptor-associated G-kinase substrate IRAG, the transcription factor TF II-I, and cysteine-rich protein 2 - latter not tested with cGK I-alpha) (71-73). The cGK isoform-specific binding suggested that interaction with an anchor protein involved the cGK N-terminus, and this was also demonstrated in several reports. There is some indication that evidence for nuclear translocation of cGK I in some cells, but not others, may depend on cell-specific anchoring of cGK I in non-nuclear sites (73). In contrast to cGK I which is considered a soluble protein in most cases, cGK II is membrane-associated via myristoylation (74), a condition essential for cGK II mediation of cGMP-dependent activation of the cystic fibrosis transmembrane conductance regulator Cl^- channel (75) and Ca^{2+} reabsorption in kidney (76). As pointed out (66), phosphorylation has been shown to constitutively activate cGK II, but by analogy to other kinases, phosphorylation might affect cGK II interactions with substrates or anchor proteins as well.

3.2.4. cGK-deficient cells, and inhibitory or inactive cGK mutants

Another complementary approach to studying cGK functions by expression of cGK is investigation of the effects of cGK deficiency. Loss of cGK I-mediated functions has been observed using cGK I-deficient platelets of chronic myelocytic leukemia patients (77), as well as smooth muscle cells, certain cGK I-containing endothelial cells, and T-lymphocytes, all of which lose cGK I during passaging in cell culture (50, 78, 79). Yet more convincing is demonstrating that a defective cGMP-dependent function can be reconstituted by specifically re-expressing cGK in deficient cells (50). Fragments or mutations of cGK designated as dominant negative forms have also been used to inhibit cGK-mediated functions. These include the regulatory regions of cGK I-alpha (59) and cGK II (63), and a catalytically inactive mutant of cGK I-alpha (Thr516A, normally phosphorylation of this threonine is essential for kinase activity) (80). Catalytically inactive mutants of cGK I-beta (Lys405Ala, the highly conserved essential lysine in the ATP-binding site of kinases) (47) and cGK II (delta-441-469, a site which contains the glycine-rich loop important for ATP binding to the kinase, just proximal to the essential lysine 482) (81) were inhibitory, but were not considered to be effective dominant negative forms.

The mechanism (82) by which mutant cGK forms act as dominant negative inhibitors of wild-type cGK forms is important to their specificity. cGK I-beta-K405A was not recommended for use as a specific dominant negative inhibitor since its inhibition could be abolished by raising the cGMP analog concentration used to stimulate wild-type cGK I (47), indicating that the inhibition was due to competition of the mutant and wild type cGKs for cGMP. Such mutants could act as a cGMP sink and consequently decrease the cGMP available for binding not only to cGK I and cGK II, but also to cGMP-regulated PDEs and

channels. This would result in global inhibition of numerous processes mediated by several cGMP targets, and thus be unspecific. The cGK I-beta-K405A mutant however did not inhibit PKA activity (47). The cGK I-Thr516Ala mutant was also suggested to act as a cGMP sink (59). The regulatory domain of cGK I-beta was also shown to inhibit the activity of both wild-type cGK I-beta and cGK II, whereas conversely the regulatory domain of cGK II inhibited only wild-type cGK II activity, very little cGK I-beta activity, and not PKA activity (63). However, the cGK II delta-441-469 mutant was shown to inhibit substrate phosphorylation by both cGK I-beta and cGK II (although not PKA), indicating that the mutant was also not a specific inhibitor of cGK II (81). The cGK II delta-441-469 mutant is, however, particularly interesting because the alternatively spliced mRNA from which it derived was found endogenously in all human, rat, and mouse tissues examined which also expressed the wild-type cGK II isoform. There are several known short forms of other kinases, produced by alternative promoters and/or splicing, which lack kinase activity and which inhibit their respective wild-type counterparts (reviewed in(81)). The splice mutant is interesting also from the standpoint that at least 15% of point mutations that result in human genetic disease cause RNA splicing defects (reviewed in (83)).

3.2.5. siRNA

Loss of function studies will undoubtedly be expanded to depletion of cellular cGK by siRNA like those now commercially available for cGK I and cGK II from Ambion (Austin, Texas). As with other methods, specificity is an important issue. Specificity is an essential determinant of whether transgenic animal gene silencing by lentiviral- (84) or adenoviral- (85) based inhibitory RNA systems can replace the more time-consuming and expensive knockout mouse model for studying defects in gene function, and also be developed for possible therapeutics purposes in humans (86). For the latter, hopes are to also employ tissue-specific promoters and inducible, for example, tetracycline-regulated promoters (84, 85). However, concerns and discussions continue about the sources of unspecificity of even short siRNAs (87, 88). The tidal wave of studies using siRNA has prompted the creation of guidelines for acceptable standards of controls for such experimentation (89). The more highly prized of these include demonstration of the same effect by two or more siRNAs and rescue experiments involving reintroducing gene expression resistant to siRNA. Another consideration regarding gene therapy with siRNA is that, although studies over the years have identified some perhaps detrimental effects of cGK I and cGK II, there are certainly a multitude of beneficial ones (1, 2) which one might want to enhance rather than silence. Since cGKs interact with certain anchor proteins (discussed above), the possibility of specific inhibition of only individual interactions might be of value. Development of such prototype peptide inhibitors designed to disrupt other protein-protein interactions for potential clinical use are already underway, e.g. for inhibiting the interaction of the tumor suppressor p53 with its endogenous inhibitor hdm2 (that targets p53 for ubiquination and degradation), to cause greater apoptosis of tumor than non-tumor cells (90). Incidentally, NO leads to phosphorylation

and stabilization of p53 (91). Stabilized p53 promotes transcription of apoptotic genes and cell-cycle regulating genes such as p21$^{WAF/CIP1}$ (90), the expression of which is also increased by NO/cGMP, apparently via PKA and cGK (92).

3.2.6. Transgenic animals

Despite longstanding fears that cGK could have many effects redundant with those of its nearest related kinase, PKA, cGK I and cGK II null mice displayed a number of defects which were obviously not compensated for by PKA. These defects will be reviewed in a separate article by F. Hofmann *et al.* (this volume) and therefore will not be duplicated here. Here, as done above for other methods, only some comments will be made pointing out a few cautions and limitations associated with transgenic mice work, although in general, generation of null mice, and isolation of cells derived from them (93), has been immensely helpful in verifying and expanding our knowledge about cGK function. Fortunately, cGK deficiency was not lethal to mice, thus compensation for any essential functions of cGK during developmental stages cannot be ruled out. Although not lethal, mice with global cGK I deficiency did have multi-system defects and were quite sick, infertile, and short-lived (40, 93-95). Certain defects appeared to be age-dependent. Evidence of hypertension in younger mice disappeared in 6-week old mice (40, 93), and although long term-potentiation (LTP) was normal in hippocampus of the global cGK I-deficient mice, it was reduced in older, longer-living, Cre-lox-generated, hippocampus-specific cGK I knockout mice (95). Consistent with the latter, LTP was similarly decreased in mice doubly mutant for endothelial NO-synthase (NOS3) and neuronal NOS1 (96). Age-dependent differences in knockout mice was also one possible explanation for the opposite results obtained by different labs with regard to the involvement of endothelial NOS3 in muscarinic, negative inotropic effects in heart (97-101). The major consensus, however, was that NOS3 is not required for muscarinic anti-adrenergic effects on cardiac contractility and L-type calcium current (LTCC). In agreement with this, it was also subsequently shown that muscarinic negative inotropic (but not NO/cGMP) effects are normal in cardiomyocyte-specific cGK I-knockout mice (102). Also, the muscarinic inhibition of LTCC was not enhanced in cardiomyocyte-specific cGK I transgenic mice (103), whereas NO/cGMP inhibitory effects mediated by cGK I were enhanced. Thus, it appears that neither NOS3 nor cGK I is involved in either muscarinic anti-adrenergic negative inotropic effects or inhibition of LTCC.

In general, despite the powerful potential of knockout technology, variable and even opposite effects of null mutations have been found with different genotype mice (104, 105). Recommendations are made for breeding strategies, spatial, tissue-specific gene deletion / conditional knockout, and phenotype rescue by re-introduction / knock-in of the wild type allele. Simple transgenic overexpression of genes by random insertion requires examination of several mouse lines in order to rule out integration effects and to evaluate effects of different transgene copy numbers. Tracking of transgenes with fluorescent tags can be useful,

and temporal control of expession via regulators such as tetracycline is sometimes necessary to avoid problems like lethality (105). In the case of transgenic tissue-specific overexpression of cGK I, expressed cGK can be activated in cells isolated from the mice, but systemic activation in the intact animal is complicated by concomitant stimulation of endogenous cGK I in other tissues. Transgenic expression of tissue-specific and tetracycline-regulated constitutive cGK I may be an alternative.

4. MONITORING ENDOGENOUS cGK I AND cGK II PRESENCE AND ACTIVITY BY SUBSTRATE (VASP AND PDE 5) PHOSPHORYLATION

Demonstration of the presence and activity of endogenous and expressed cGK in cells is a basic, but important, aspect of attributing functions to cGK. This is particularly the case for endogenous cGKI and cGK II, as mentioned above, since they are lost in many primary cell types upon passaging in cell culture and are undectectable in many cell lines (43, 50, 78, 79). However, quite often cGK presence or activity is only presumed on the basis of cellular responses to agents like those shown in Table 1, despite overlapping targets and the fact that the data in Table 1 were acquired *in vitro* and cannot adequately predict the complex effects which have been observed with some agents in intact cells. In addition, the validity of certain inhibitors such as KT5823 has been seriously compromised by reports of their variable quality and unspecific effects. Some batches of KT5823 were inactive in controlled *in vitro* assays of cGK activity (28, 106), did not inhibit 8CPT-cGMP effects in platelets and mesangial cells, and even had stimulatory effects in platelets (28). In fact, KT5823 had greater inhibitory effects on other kinases tested in *in vitro* assays (32). H89 effectively inhibits quite a number of kinases *in vitro* (29), perhaps an explanation for erratic effects of H89 observed with intact cells (30). Unfortunately the protein kinase inhibitor (PKI) which is a quite good inhibitor of PKA, but not cGK (33), was not tested in the Davies *et al.* screen (29). Lack of inhibition by PKI has often been used to confirm that a proposed cGK effect is not mediated by PKA. However, inhibitory properties of PKI peptides depend on peptide length, amidation, etc. (33). Recent studies using adenoviral vector expression of full length PKI-alpha in cells demonstrated its inhibition of PKA but not cGK (30). PKI not only inhibits PKA but acts as a chaperone for nuclear export of PKA C subunit from the nucleus to the cytosol. It should be noted however, that the phenotype of PKI-alpha null mice was nevertheless unexpected, suggesting other possible, yet undescribed actions of PKI (107). Unspecific effects of several Rp-cGMP analogs have also been noted recently in both human and mouse platelets (108, 109), discussed at the end of section 4.1. The newer cGK inhibitor peptides, DT-2 and DT-3 (34, 35), look very promising in *in vitro* assays and limited intact cell studies, but are subject to some reservations about their performance in a wider variety of cell types. Until information is published with more widespread cell types, all inhibitors, including the DT inhibitors, should be used under carefully controlled conditions to ascertain their specificity and effectivity in new systems, and their use should contribute only one of

several pieces of evidence necessary to conclusively propose a role for cGK in a given cellular function. Some misleading data, as discussed below, can thus be potentially avoided.

4.1. Intracellular VASP phosphorylation

The presence of cGK can be determined in cells by specific antibodies, however it is optimal to be able to assess intracellular activation of cGK and its correlation with functions. Activation of both cGK I (47) and cGK II (49, 81) can be analyzed by intracellular phosphorylation of the substrate VASP (110). VASP has three sites (Ser157, Ser239, and Thr278 in human) phosphorylated by both PKA and cGK, whereas Ser157 is phosphorylated more rapidly by PKA and Ser239 more rapidly by cGK in platelets (111), a difference which can be less pronounced in other cell types (47, 50). Striking cell type differences have also been observed in the time course (5 min versus at least 2 h duration) of VASP phosphorylation in response to sodium nitroprusside (50), suggested by other results to perhaps derive from highly efficient cGMP-hydrolyzing PDE(s) in T-lymphocytes in comparison to platelets.

Examination of VASP phosphorylation (see Figure 1), was initially performed using a polyclonal antibody (e.g. M4, later also IE273) which recognized only phosphorylation of the VASP Ser157 site. The polyclonal antibody recognized both phospho- and dephospho- VASP, and detected phosphorylation of Ser157 in SDS/PAGE Western blots by a shift in VASP mobility from Mr 46 kDa to 50 kDa. Subsequently, specific monoclonal antibodies recognizing only VASP phosphorylated at either Ser239 (16C2) or Ser157 (5C6) were developed (47, 111). The 5C6 antibody recognizing VASP-P-Ser157 detects a 50 kDa protein, whereas the 16C2 antibody recognizing VASP-P-Ser239 detects a 46 kDa protein, as well as a 50 kDa protein if VASP-P-Ser239 is additionally phosphorylated on Ser157 and shifts to the higher Mr. The monoclonal antibodies were made in collaboration with the company nanoTools (Teningen, Germany) which markets them for research use. Although some publications continue to equate the shift to the higher Mr of 50 kDa with VASP phosphorylation only by PKA, this is not exclusively the case, since cGK also phosphorylates Ser157 and causes the shift with different efficiency in various cell types (47, 50, 111), depending perhaps on differences in the presence of at least three cellular phosphatases shown to dephosphorylate VASP sites *in vitro* (112). The VASP Thr278 site has not been extensively studied since it appears to be a weaker substrate for both PKA and cGK than the other two sites (113), and there has been some speculation about whether it has some endogenous basal phosphorylation which interferes with attempts to detect stimulation of its phosphorylation in cells. However, mutation experiments suggested that this site was a co-determinant of the localization of VASP in focal adhesions in endothelial cells (47).

Since both PKA and cGK phosphorylate VASP on both Ser239 and Ser157, albeit with somewhat different and cell-type dependent kinetics, care must be taken to determine
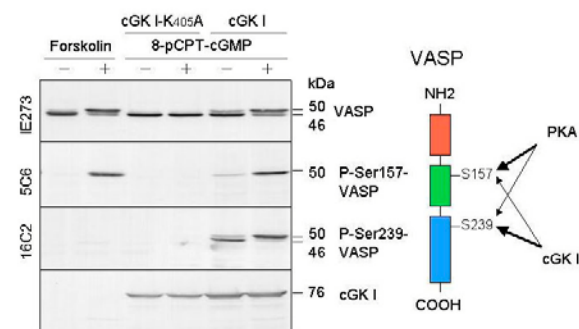
Figure 1. Adenovirus-mediated expression of cGK I-beta and demonstration of cGK activity as cGMP-dependent phosphorylation of endogenous VASP in HUVEC. Subconfluent HUVEC (passages 1-3) were either uninfected, or infected with equal amounts (5 x 10^9 particles/ml) of adenoviral vectors for either wild-type cGK I-beta (cGK I) or a catalytically inactive mutant of cGK I-beta (cGK I-K405A). Cells were incubated in the absence or presence of either 3 μM forskolin for 10 min (uninfected cells), or 100 μM 8-pCPT-cGMP for 5 min (cells one day post-infection). Cell homogenates were analyzed on Western blots labeled with specific antibodies recognizing either total VASP and VASP phosphorylation of Ser 157 as a shift in VASP apparent Mr from 46 kDa to 50 kDa (IE273 Ab, upper panel), VASP phosphorylated on Ser 157 (5C6 Ab, second panel from top), VASP phosphorylated on Ser 239 (16C2 Ab, third panel from top), or cGK I (bottom panel). Uninfected HUVEC contained no detectable endogenous cGK I (bottom panel, lanes 1 and 2), and VASP phosphorylation on Ser 157 was detected in these cells only in response to forskolin (lane 2), but not 8-pCPT-cGMP (data not shown). The lack of Ser239 phosphorylation by forskolin (third panel, lane 2) is unexpected since P-Ser239-VASP was observed after forskolin and prostaglandin treatment of other cells (28, 111), thus cell-type specific differences may be involved. In adenoviral vector infection studies of HUVEC, the control of catalytically inactive cGK I-K405A (lanes 3 and 4) gave no VASP phosphorlyation of Ser 157 (upper two panels) or Ser 239 (third panel from top) in response to 8-pCPT-cGMP (lane 4). In contrast, expressed wild type cGK I showed some basal activity (lane 5) which was further stimulated by 8-pCPT-cGMP (lane 6) with regard to both Ser 157 (upper two panels) and Ser 239 phosphorylation (third panel from top). Phosphorylation of VASP on Ser 239, either without (46 kDa band), or with (50 kDa band) concomitant phosphorylation of Ser 157, was detected by the 16C2 Ab. Data adapted from (47).

which kinase mediates the intracellular effects of cGMP. cGMP signals can be mediated by PKA (114) through either of two different mechanisms, by inhibition of PDE 3 (discussed above in section 3.2.1) at concentrations similar to those that activate cGK (8, 37, 38) to increase cAMP and its activation of PKA, or possibly by direct activation of PKA under certain circumstances. Compared with cGK, much higher levels of cGMP (somewhat lower concentrations of some cGMP analogs) are required to directly activate PKA (8) (see Table 1). Studies of smooth muscle and platelets from cGK I-deficient mice (40, 115),

cGK I-deficient platelets from chronic myelocytic leukemia patients (77), as well as endothelial cells and T-lymphocytes which lost cGK I upon passaging in cell culture (50, 79), suggest that PKA is not involved in NO/cGMP signaling under physiological conditions, although PKA could come into play in pathophysiological settings such as inflammatory conditions (116) and septic shock when high NO/cGMP levels are produced (93). In cases in which VASP phosphorylation was studied, cGMP-dependent, but not cAMP-dependent phosphorylation was absent in cGK I-deficient systems (47, 50, 77, 79, 115), also suggesting that cGMP effects were not being mediated by PKA.

A vast amount of data supports an inhibitory role of cGMP/cGK I in platelet activation and aggregation (117). However, recent results proposed a radically different view, that cGMP has biphasic effects in platelets, promoting platelet activation at early times via cGK I (118), before inhibiting platelets at later times via PKA (119). These conclusions relied partly on the use of activators and inhibitors of cGK and PKA which this review has suggested harbors inherent risks, but some conclusions were also based on experiments with cGK I-deficient mice (118) and assessment of intracellular cGK activity by analysis of VASP phosphorylation (118, 119). The conclusions were later shown to be invalid by carefully controlled experiments, including ones again using the same cGK I-deficient mice (109), as well as analysis of VASP phosphorylation (108, 109), illustrating that methodologies alone cannot guarantee infallible and carefully interpreted results, but require judicious use for reaching the best conclusions. These latter experiments also yielded further insight into unspecific effects observed with cGMP analogs used as activators and inhibitors of cGK in platelets. cGK **in**dependent functional effects were observed 1) in response to cGMP analogs in platelets from cGK I-deficient mice (109) and 2) in human platelets in which the same platelet response was observed with both activators and inhibitors of cGK I, whereas only cGK activators stimulated VASP phosphorylation (108). 3) Also, an analog (Rp-8-Br-PET-cGMPS) designated as a cGK inhibitor was effective at a concentration (1 μM or less) which is far below that of cGK in platelets (14 μM cGMP binding sites per cGK dimer (120), lower in other cell types (121)), and thus most likely inadequate to appreciably activate cGK. 4) Some functional effects could even be elicited by equivalent noncyclic guanosine monophosphates (e.g. 8-Br-GMP compared to 8-Br-**c**GMP), clearly an indication that cGK I was not involved (108). 5) Furthermore, extracellular application of certain cGMP analogs had effects on platelets at timepoints earlier than could be expected based on their stimulation of VASP phosphorylation by intracellular cGK (108). Platelet activating effects of cGMP analogs were reported after no or minimal preincubation time with cGMP analogs (119), strongly suggesting that the effects were extracellular, possibly on membrane receptors, channels, etc. The greater implication of the flawed data proposing cGMP involvement in platelet activation was that potency drugs such as sildenafil (Viagra) which increase cGMP carry a potential risk of thrombosis (119), a contention which is

untenable based on current knowledge from the follow-up studies (108, 109).

4.2. Intracellular PDE 5 phosphorylation

In addition to VASP phosphorylation, PDE 5 phosphorylation has more recently been characterized as an effective monitor of intracellular cGK activation. PDE 5 is phosphorylated by both PKA and cGK *in vitro*, but primarily by cGK in intact cells (36, 122). Phosphorylation of PDE 5 is tightly regulated by cGMP binding to allosteric sites which then exposes Ser102 (in human PDE 5) for phosphorylation. In platelets from cGK I-deficient mice, NO-stimulated PDE 5 phosphorylation was reduced 75%, the remaining 25% postulated to result from PKA-dependent phosphorylation, and similarly VASP phosphorylation was also partially preserved (123). One could speculate that the residual phosphorylation could be explained by NO inhibition of PDE 3 to stimulate cAMP and PKA. Residual PKA-dependent VASP phosphorylation was considered less pronounced when 8-pCPT-cGMP was used with cGK I-deficient platelets (115). Because 8-pCPT-cGMP has far less effect than cGMP on PDE 3 (see Table 1), this could account for less residual VASP phosphorylation by PKA in 8-pCPT-cGMP stimulated platelets (115) compared to NO/cGMP stimulated ones (123). In other studies, a high concentration (1 mM) of 8-Br-c<u>A</u>MP alone showed no detectable PDE 5 phosphorylation in platelets of either wild type or cGK-deficient mice, suggesting that PKA was not phosphorylating PDE 5 (36, 122). However, some PDE 5 phosphorylation was observed in the presence of 1 mM 8-Br-cGMP together with 1 mM 8-Br-cAMP. One can speculate whether this could be explained by 8-Br-cGMP inhibition of a PDE which hydrolyzes 8-Br-cAMP, such that the latter activates PKA more strongly than when present alone.

4.3. Tissue and cell type suitability for VASP and PDE 5 phosphorylation studies

The choice of analyzing VASP versus PDE 5 phosphorylation in certain cells may need to be based on empirical testing and on the relative levels of cGK and VASP or PDE 5, the degree of basal or background phosphorylation present which may hinder detection of further stimulation, etc. PDE 5 phosphorylation has been noted to be useful in smooth muscle (mouse and human uterus and mouse lung greater than mouse aorta), and in human and mouse platelets, and mouse cerebellum (36, 122-124). In cerebellar Purkinje cells, PDE 5 phosphorylation was also used to demonstrate the presence of cGMP elevations needed to stimulate cGK, since previously cGMP has been difficult to detect (124). Until now, VASP phosphorylation has been studied primarily in human, rabbit, and rat vasculature (125-130), human and mouse platelets (77, 115, 131), human T-lymphocytes (50), human endothelial cells (only certain ones containing cGK I, not human umbilical vein endothelial cells (HUVEC) in our studies, e.g., Figure 1) (47, 79), human dermal fibroblasts (47), rat adrenal zona glomerulosa cells (49), rat kidney mesangial cells (28), mouse cardiac fibroblasts (132), and neonatal rat cardiac myocytes (133), although the level in adult myocytes is difficult to work with. Substantial amounts of VASP itself were also observed in mouse neonatal and adult lung, spleen, and stomach, as well as neonatal brain and heart and adult large intestine (134). Although most studies of VASP phosphorylation have involved the use of antibodies in Western blots, the antibodies have also been used in FACS flow cytometry analysis (135-137) and in immunocytochemistry (130).

4.4. Monitoring function and dysfunction of signaling pathways using VASP phosphorylation

In addition to the extensive use of P-VASP antibodies to verify the presence and activation of cGK in intact cells as discussed above, they have been used for monitoring the physiological effects as well as dysfunction and pathology of signaling pathways, and also for evaluating responses to therapeutic regimens. A good illustration is the usefulness of VASP phosphorylation for tracing the effects of vascular endothelial cell factors and therapeutic agents on platelets and vasculature. Endothelial cell derived inhibitors of platelet activation, i.e. prostaglandins and NO, increase cAMP and cGMP, respectively, and cause platelet VASP phosphorylation. Platelet activation by ADP via purinergic Gq/Gi-coupled P2Y12 receptors and a G_i-protein is inhibited by NO (138), as well as by the antiplatelet drug clopidogrel (135, 139). Clopidogrel given to human volunteers abolished the inhibitory effects of ADP/P2Y12 on prostaglandin E_1-stimulated, cAMP-dependent phosphorylation of VASP (135, 139). Platelets are a major determinant of intracoronary artery thrombosis, stenosis, and re-stenosis after stent correction, however thrombotic risk has been dramatically reduced with the use of clopidogrel. Despite this, there still exists a finite risk of stent thrombosis in 1-2 % of cases, with devastating effects (reviewed in (136)). In a small-scale study, FACS analysis indicated significantly lower VASP phosphorylation in platelets from patients who experienced subacute thrombotic events 30 days post coronary stenting compared to those who did not, and patients could be classified as good, delayed, and bad responders to clopidogrel (136). Larger studies are needed to demonstrate the full clincial value of monitoring VASP phosphorylation to identify patients at risk for post-coronary stent complications and in need of prophylactic measures to prevent it.

FACS analysis of platelets from human volunteers has shown that acute inhibition of endogenous NO production decreased VASP phosphorylation and caused rapid platelet activation, whereas sublingual nitrate normalized this (137). This is the first real *in vivo* evidence of measurable biochemical effects of NO on platelets. Further evidence suggests that cGK I mediates effects of dipyridamole, which, in combination with low-dose aspirin, is very effective in preventing recurrent stroke; dipyridamole, an inhibitor of cGMP hydrolysis by PDE 5, increases VASP phosphorylation, and enhances antiplatelet effects of NO, but not those of cAMP (140). Cerebral and peripheral thromboembolic events also contribute to the high morbidity in congestive heart failure (CHF) (141). In a rat model of chronic myocardial infarction-induced CHF, blockade of the renin-angiotensin-aldosterone system, using an aldosterone receptor antagonist and an angiotensin-converting enzyme inhibitor, restored endothelium-dependent.
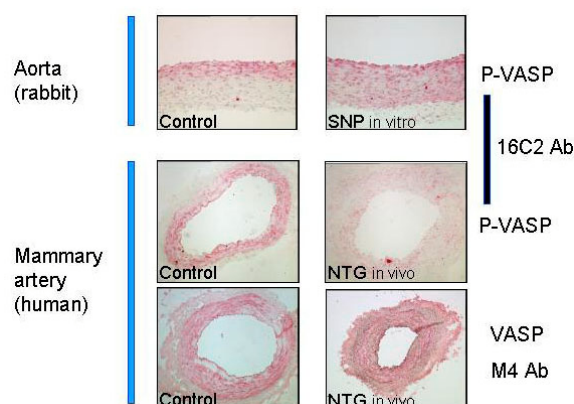
Figure 2. Immunocytochemical staining of Ser239-VASP phosphorylation (16C2 antibody). In rabbit aorta, increased P-VASP is observed after *in vitro* incubation with 10 µM sodium nitroprusside (SNP) (upper panels, right versus left). In human mammary artery, total VASP protein (M4 antibody) is shown to be similar under conditions both before (control) and after nitroglycerin (NTG *in vivo*) infusion (0.5 µg/kg per min) for 24-48 h prior to bypass surgery (bottom panels). Under these same conditions, P-Ser239-VASP is greatly decreased after NTG treatment (middle panels, right versus left), signifying the suppression of endogenous vasodilator (e.g. NO) effects and the development of tolerance to NTG as superoxide production increased; the expression of sGC and cGK I remained unchanged (not shown). Data adapted from (130), using additional unpublished data (rabbit aorta) from Maria Wendt and Thomas Münzel (Hamburg).

NO-bioavailability and VASP phosphorylation, and attenuated platelet activation and the hypercoagulable state (142).

Endothelial dysfunction, an important problem in cardiovascular medicine, and a common feature of hypercholesterolemia/lipidemia (125-127), hypertension (128), and nitrate tolerance (129, 130) (see Figure 2), has also been effectively monitored using VASP phosphorylation. This is a significant achievement since changes in other individual upstream components (endothelial NOS3, soluble guanylate cyclase(sGC), PDE) of the NO/cGMP signaling pathway have not been consistently reliable predictors of endothelial dysfunction (reviewed in (2)). In many of the examples of endothelial dysfunction there is scavenging of NO by reactive oxygen species and often subsequent multiple alterations or compensatory changes in components of the NO/cGMP signaling pathway, and NO tolerance. Because cGK I occupies a very distal position in this signaling chain, its activity with regard to VASP phosphorylation seems to reflect the sum of changes in activity and/or expression of upstream components as well as cGK I itself. A series of relatively new compounds developed at Bayer and Aventis are NO-independent activators of sGC that do not invoke tolerance, and in model systems demonstrate antiplatelet, antihypertensive, and cardiorenal properties which show promise for the treatment of cardiovascular

diseases including CHF (143-145).

5. SUMMARY AND PERSPECTIVE

The authenticity of synthetic agents as regulators of cGK certainly needs to be validated in the specific study of interest. Beyond this, the analysis of cGK functions has been facilitated by major technological advances which nevertheless also demand use in carefully controlled experiments. Despite caveats, the fund of information and technology currently available should enable the design of experiments which reliably convey effects of cGK rather than other targets. Briefly, some criteria for identifying functions of cGK are:

1) The function can be elicited by an appropriate physiological agent such as NO or NPs which increase cGMP.
2) The presence and activity of cGK can be demonstrated (e.g. by use of cGK antibodies in Westerns and analysis of VASP or PDE 5 phosphorylation) in intact cells displaying the function in response to NP or NO, etc.
3) Activators or inhibitors of cGK mimic or block NO/NP effects in intact cells. Indications of cGK independent effects such as a) occurance at a time too early for the agent to have intracellular effects, b) occurance at a concentration far below that of cGK, or c) occurance in response to both a cGMP analog and its corresponding noncyclic guanosine monophosphate, should be heeded.
4) NO/NP effects can be mimicked by constitutively active cGK or transgenic expression of cGK but not catalytically inactive cGK, or conversely can be inhibited by loss of cGK from cells (culturing or siRNA) or deficiency in mice.

In addition to defining cGK actions, a further challenge for the future will be to understand and find ways of intervening in GK interactions with signaling complexes via specific proteins. This could offer the chance to alter selected cGK functions without having global effects on several of its functions, thus discriminating between beneficial and detrimental GK effects. Analysis of cGK activity also has an imminent diagnostic potential. Present studies focus on the analysis of cGK-dependent VASP phosphorylation in whole blood, both to expedite clinical monitoring of diminished antiplatelet/antithrombotic capacity of the endothelium, a harbinger of cardiovascular disease, as well as to evaluate therapeutic regimens designed to alleviate such maladies.

6. ACKNOWLEDGEMENT

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Abbreviations: cGK: cGMP dependent protein kinase, CHF: congestive heart failure, HUVEC: human umbilical vein endothelial cells, LTCC: L-type calcium current, NO: nitric oxide, NOS: nitric oxide synthase, NP: natriuretic peptide, NTG: nitroglycerin, PDE: phosphodiesterase, PKA: cAMP dependent protein kinase, P2Y12: purinergic Gq/Gi-coupled receptor, sGC: soluble guanylate cyclase, siRNA: small interfering RNA, VASP: vasodilator stimulated phosphoprotein

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Send correspondence to: Dr Suzanne M. Lohmann, Institute of Clinical Biochemistry and Pathobiochemistry, University of Wuerzburg Medical Clinic, Josef Schneider Str. 2, 97080 Wuerzburg, Germany, Tel: 49-931-201-45144, Fax: 49-931-201-45153, E-mail: slohmann@klin-biochem.uni-wuerzburg.de