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Review

Physiology and pathophysiology of vascular signaling controlled by guanosine 3′,5′-cyclic monophosphate-dependent protein kinase★

Ingvild Birschmann and Ulrich Walter✉

Institut für Klinische Biochemie und Pathobiochemie, Universitätsklinikum Würzburg, D-97078 Würzburg, Germany

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Recent medical advances suggest that the cellular natriuretic peptide/cGMP and NO/cGMP effector systems represent important signal transduction pathways especially in the cardiovascular system. These pathways also appear to be very interesting targets for the possible prevention of cardiovascular diseases. Exciting candidates for prevention include cGMP-dependent signaling networks initiated by natriuretic peptides (NP) and nitric oxide (NO) which are currently explored for their diagnostic and therapeutic potential. cGMP signaling contributes to the function and interaction of several vascular cell types, and its dysfunction is involved in the progression of major cardiovascular diseases such as atherosclerosis, hypertension and diabetic complications. This review will take a focussed look at key elements of the cGMP signaling cascade in vascular tissue. Recent advances in our knowledge of cGMP-dependent protein kinases (cGK, also known as PKG), the potential for assessing the functional status of cGMP signaling and the possible cross talk with insulin signaling will be reviewed.

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✉Corresponding author: Ulrich Walter, Institut für Klinische Biochemie und Pathobiochemie, Universitätsklinikum Würzburg, D-97080 Würzburg, Germany; tel.: (49 931) 20145144/45479; fax: (49 931) 20145153; e-mail: uwalter@klin-biochem.uni-wuerzburg.de

Abbreviations: sGC, soluble guanylate cyclases; cGK, cGMP-dependent protein kinase; MLC, myosin light chain; NOS, NO synthase; PDE, phosphodiesterase; SNP, sodium nitroprusside; VASP, vasodilator-stimulated phosphoprotein; VSMC, vascular smooth muscle cell.

Abnormalities in endothelial and vascular smooth muscle cell function, as well as pathological activation of human platelets, play a decisive role in atherosclerosis and its complications (Hu *et al.*, 2002; Bhatt & Topol, 2003). These factors contribute to the pathogenesis of cardiovascular and cerebrovascular diseases which remain the major cause of death in most industrialized countries. Substantial efforts were undertaken in the last decades to elucidate underlying mechanisms, and some progress has been made.

Thirty years ago Schultz *et al.* (1977) and Katsuki *et al.* (1977) described the stimulation of soluble guanylate cyclases (sGC) by both NO and organic nitrates. The discovery of NO as "endothelium-derived relaxing factor" (EDRF) that regulates vascular tone was a major advance in this field (Furchgott & Zawadzki, 1980; Ignarro *et al.*, 1987; Palmer *et al.*, 1987). NO is synthesized by NO-synthases (endothelial eNOS, inducible iNOS, neuronal nNOS) from L-arginine. NO induces activity of the target enzyme sGC, which results in increasing tissue levels of the second messenger cGMP. cGMP activates cGMP- dependent protein kinase (cGK) that promotes vasorelaxation *via* phosphorylation of proteins that regulate intracellular Ca^{2+} levels. In addition to these effects on vascular smooth muscle, the NO/cGMP/PKG pathway affects various heart functions, platelets, immune cells and neurotransmission (von der Leyen & Dzau, 2001). Therefore, in essentially all mammalian cells this pathway is fundamental and highly regulated. Current research focuses on various steps of this signaling pathway with the aim to use this knowledge for better diagnostics and therapeutic applications.

cGMP/cGK

Two pathways regulate the synthesis of cGMP. In the first one, cGMP is generated from GTP in response to different natriuretic peptides (NP), namely the atrial (ANP), B-type (BNP) or C-type (CNP) natriuretic peptides. In the second pathway, cGMP is synthesized by soluble guanylyl cyclase in response to NO as mentioned above.

Two mammalian genes and three isotypes of cGK have been described so far:

- type I cGK consists of an α and a β isoform, splice variants of a single gene. It is also identified as the most prominent cGK isotype in the cardiovascular system. Very high levels of cGKI are found in vascular smooth muscle cells, endothelial cells and platelets.
- type II cGK is mainly expressed in intestine, kidney and brain.

cGK signaling plays an important role in vascular biology, in regulating smooth muscle tone and in proliferation and differentiation of vascular smooth muscle cell (VSMC) (Ruth, 1999; Lincoln *et al.*, 2001). In addition, it also regulates endothelial cell (i.e. permeability and motility) and platelet function (Lohmann *et al.*, 1997; Ruth, 1999; Munzel *et al.*, 2003). Further insight into the function of the two protein kinases has been obtained by the analysis of their substrates and by the generation of mice deficient in either cGK I or II. Homozygous deletion of the cGKI gene in mice abolishes NO/cGMP-dependent relaxation of vascular (Pfeifer *et al.*, 1998), visceral (Pfeifer *et al.*, 1998; Ny *et al.*, 2000) and penile smooth muscle (Hedlund *et al.*, 2000), resulting in severe vascular and intestinal dysfunction with death at an early age (Pfeifer *et al.*, 1998). Additionally, the knockout mice showed impaired NO/cGMP-dependent inhibition of platelet activation (Massberg *et al.*, 1999).

The phenotype of cGKII knockout mice included normal lifespan (Pfeifer *et al.*, 1996), decreased longitudinal bone growth (Pfeifer *et al.*, 1996), decreased intestinal chloride secretion (Pfeifer *et al.*, 1996; Vaandrager *et al.*, 2000) and altered renin secretion (Wagner *et al.*, 1998).

Results obtained with knockout mice discussed here support that cGKI plays an im-

portant role in the cardiovascular system. It also seems that cGKII has an influence on haemodynamic parameters *via* regulation of renin release and ion transport in the kidney.

cGK in different cell types has numerous effects. In the next section we discuss two of the best characterized pathways and examples of NO/cGMP/cGK signaling.

ROLE OF cGMP SIGNALING PATHWAY IN VSMC

The serine/threonine protein kinase cGK mediates VSMC relaxation by catalyzing the phosphorylation of specific substrate proteins (Munzel *et al.*, 2003). Many of the cGK substrate proteins, that have been implicated in relaxation, are involved in reducing intracellular Ca^{2+} concentration or lowering the Ca^{2+} sensitization. Both effects result in inhibition of myosin light chain (MLC) phosphorylation and therefore cause smooth muscle cells (SMC) contraction (Ammendola *et al.*, 2001; Lincoln *et al.*, 2001; Pfitzer, 2001). Reduced intracellular Ca^{2+} concentration occurs due to various actions like Ca^{2+} release from intracellular stores, Ca^{2+} uptake mediated by calcium adenosine 5′-trisphosphatase of the endoplasmatic reticulum (ER) and Ca^{2+} influx. Phosphorylation of IRAG ($InsP_3R$ associated cGMP kinase substrate; $InsP_3R$: $InsP_3$ receptor; $InsP_3$: inositol 1,4,5-triphosphate) (Schlossmann *et al.*, 2000) by cGKI results in inhibition of IP_3/IRAG-mediated Ca^{2+} release (Ammendola *et al.*, 2001) from the ER and therefore in activation of MLC kinase and vasoconstriction. Ca^{2+} uptake is normally mediated by calcium ATPase which could be inhibited by cGKI-dependent phosphorylation of phospholamban. cGKI also phosphorylates a BK_{Ca} channel, which leads to K^+ efflux from the cell, hyperpolarization, inhibition of Ca^{2+} entry through voltage-dependent ion channels and relaxation (Fukao *et al.*, 1999; Hofmann *et al.*, 2000; Lincoln *et al.*, 2001).

In summary, a number of cGKI-dependent mechanisms have been identified that regulate the cytosolic Ca^{2+} concentration. However, the precise physiological *in vivo* significance of these different pathways is still debated.

cGKI apparently also affects smooth muscle tone by reducing the Ca^{2+} sensitivity of the contractile apparatus (Ruth, 1999). cGK phosphorylation of RhoA at Ser 188 induces RhoA translocation from the membrane to the cytosol whereby RhoA is inactivated. Thus it inhibits Rho kinase and consequently activates MLC phosphatase to dephosphorylate MLC. Consequently, cGKI-mediated activation of MLC phosphatase and calcium desensitization might involve inhibition of Rho/Rho kinase-dependent and -independent pathways (Sauzeau *et al.*, 2000; Etter *et al.*, 2001).

Other studies have suggested that NO and cGMP inhibit VSMC proliferation, a hallmark of many vascular disorders (Young *et al.*, 2000).

ROLE OF cGMP SIGNALING PATHWAY IN PLATELETS

Over the past decade the role of platelets evolved from passive participants in the coagulation cascade to that of active synthesizer of humoral factors that potentiate both clot formation and inflammation. Antiplatelet therapy is presently the golden standard in the secondary prevention of cardiovascular incidents (Bhatt & Topol, 2003). Dipyridamole – an enhancer of NO/cGMP/cGK signaling pathway – is one of the very effective drugs (in combination with aspirin) in the secondary prevention of ischemic strokes again demonstrating the importance of this pathway for platelet activation/aggregation.

In vivo, platelets are continually exposed to the endothelial-derived factors NO and prostacyclin ($PG\text{-}I_2$), which inhibit and limit unwarranted platelet activation by increasing

the level of intracellular cAMP and cGMP (Nolte *et al.*, 1994; Schwarz *et al.*, 2001) (Fig. 1). Elevation of cGMP, induced by NO or pharmacological drugs such as the antihypertensive, NO-releasing agent sodium nitroprusside (SNP) and the cGMP-phosphodiesterase type 5 (PDE 5), has been recognized to cause inhibition of platelets (Bhatt & Topol, 2003; Aktas *et al.*, 2003). One crucial effect is again the activation of cGK and the subsequent phosphorylation of specific target proteins such as the vasodilator-stimulated phosphoprotein (VASP).

Agonists which stimulate platelets such as thrombin, ADP, thromboxane receptor agonists and collagen (Haslam *et al.*, 1978) are also known to moderately raise platelet cGMP levels and possibly to limit and counteract the extent of platelet activation. However, recent published data suggest a stimulatory role of cGMP and cGK in von Willebrand factor (vWF)- and thrombin-induced platelet activation. Li *et al.* (2003a, 2003b) report a stimulatory effect on MAP kinases and the fibrinogen receptor by activating cGK. Two groups, S. Watson's group and our own group, could not confirm these results. Our group (Gambaryan *et al.*, 2004) demonstrated a potent cGK-unrelated inhibition of human platelet activation by both cGK activators and inhibitors, using the conditions published by Li *et al.* (2003a, 2003b).

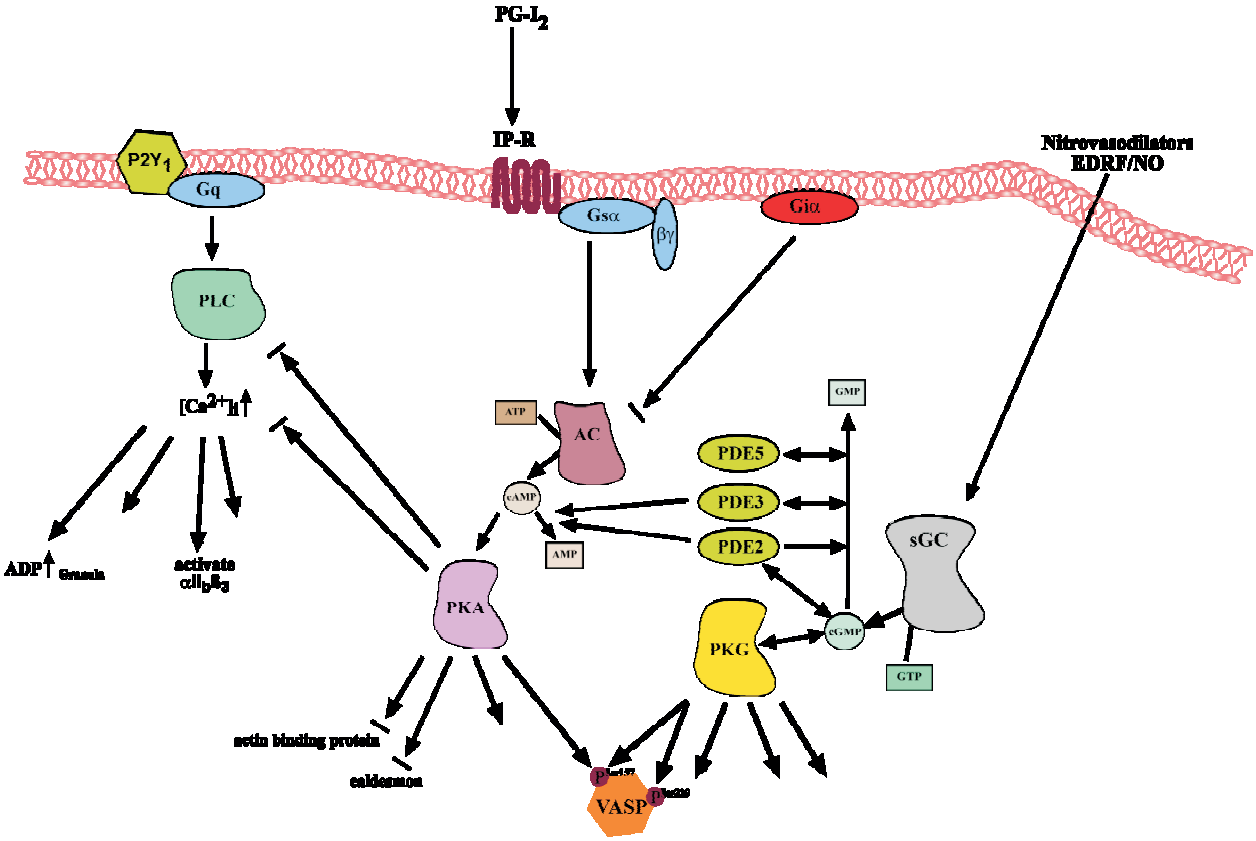

Figure 1. NO/cGMP/cGK-mediated mechanisms for platelet activation.

cGKI affects diverse signaling pathways leading to platelet activation. Abbreviations: AC, adenylate cyclase; EDRF, endothelium-derived relaxing factor; IP-R, prostaglandin receptor; PG-I_2, prostaglandin I_2; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase (cGK); PLC, phospholipase C; sGC, soluble guanylate cyclase; VASP, vasodilator-stimulated phosphoprotein.

Further evidence against a cGK-mediated activation of MAP kinases and $\alpha II_b\beta 3$ integrin by vWF was reported by Marshall *et al.* (2004). They demonstrated a critical role of Src kinases but not MAP kinases in the vWF-dependent activation and also confirmed the inhibitory role for cGMP-elevating agents in regulating this process.

cGMP AND INSULIN

The above comments summarized some current evidence that the NO/cGMP/cGK signaling pathway is important in the control of many cellular functions. VSMCs are the major constituents of blood vessel walls and are responsible for the maintenance of vascular tone. These cells also contribute to the pathogenesis of type 2 diabetes, hypertension, and cardiovascular diseases (Cohen & Vanhoutte, 1995; Sowers & Epstein, 1995; Sowers *et al.*, 2001; Gewaltig & Kojda, 2002). In these diseases increased contractility of VSMCs, an abnormal vascular tone and defective vasorelaxation are the earliest abnormalities observed. In this section we will therefore briefly review the relationship of the NO/cGMP/cGK signaling pathway and type 2 diabetes.

Insulin can inhibit VSMC contraction, migration and growth in the normal vasculature (Somlyo & Somlyo, 1994; Hsueh & Law, 1999). Failure of this function in insulin-resistance may contribute to enhanced atherosclerosis/restenosis in these clinical cases. Studies with human subjects indicate that hypertension and type 2 diabetes are associated with an impaired vasodilatory response to acetylcholine and SNP, suggesting impaired VSMC responsiveness to NO (Steinberg *et al.*, 1996). A relationship of impaired endothelium-dependent relaxation and expression levels of NOS in diabetic mice was shown (Gunnett *et al.*, 2003).

Recent studies demonstrated that insulin induces relaxation of VSMC *via* stimulation of myosin phophatase and inhibition of Rho kinase activity (Sandu *et al.*, 2000; Begum *et al.*, 2000). Further data revealed that insulin inhibits Rho signaling by altering post-translational modification of RhoA *via* nitric oxide/cGMP signaling pathway causing myosin-bound phosphatase (MBP) activation, actin cytoskeletal disorganization and vasodilatation (Begum *et al.*, 2000). These effects may contribute to the well-known vasodilator actions of insulin. Therefore, an evaluation of platelet function during development of diabetes is of major interest. Previous studies (Tschoepe *et al.*, 1993) showed that platelets in diabetic patients are larger than those of healthy patients. In addition, platelets of diabetics are characterized by an enhanced activation and aggregation in response to different stimuli *in vitro*. Also, recently an altered platelet NO level and generation was observed in platelets from healthy donors after insulin-treatment (McKendrick *et al.*, 1998; Fleming *et al.*, 2003). However, the underlying mechanisms and their contribution to the development of cardiovascular diseases have not as yet been established.

Given the importance of the NO/cGMP/cGK signaling pathway for the cardiovascular system and effects on platelet function as described above, an understanding of the exact mechanism of insulin function and the pathophysiology of the vascular complications associated with diabetes promises to be a good setting-out point for achieving more effective therapeutic tools.

Although much progress has been made in recent years regarding the NO/cGMP/cGK signaling pathway and the effects of different diseases, functional *in vivo* data is often lacking. Hopefully over the next few years progress in molecular and biophysical approaches will give us a clearer picture of the function and regulation of cGK, allowing many important questions to be addressed and also to see profound advancement of further therapies in treating vascular signaling.

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