Minireview

The Biology of Cyclic GMP-dependent Protein Kinases

Franz Hofmann
From the Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedeletterstrasse 29, D-80802 München, Germany

Basic Properties of cGMP-dependent Protein Kinase Isoforms

cGKs^1 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 (1, 2). Mammals have two cGK genes, prkg1 and prkg2, that encode cGKI and cGKII. The N terminus (the first 90–100 residues) of cGKI is encoded by two alternatively spliced exons that produce the isoforms cGKlα and cGKlβ. The enzymes have a rod-like structure and are activated at submicromolar to micromolar concentrations of cGMP (3, 4). They are composed of three functional domains: an N-terminal (A) domain, a regulatory (R) domain, and a catalytic (C) domain (for details see Refs. 1 and 2). The regulatory domain contains two tandem cGMP-binding sites that interact allosterically and bind cGMP with high and low affinity. Occupation of both binding sites induces a large change in secondary structure (5) to yield a more elongated molecule (6, 7). 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 site and allows MgATP-dependent autophosphorylation. Activation of heterophosphorylation may be preceded by autophosphorylation. Autophosphorylation increases the spontaneous activity of cGKI and cGKII (8–11) and is initiated by the binding of low cGMP concentrations to the high affinity site of cGKI (12, 13). In addition to controlling activation and inhibition of the catalytic center, the N terminus has two other functions: dimerization, cGKs are homodimers that are held together by a leucine zipper present in the N terminus, and targeting, the enzymes are targeted to different subcellular localizations by their N termini.

Tissue Distribution

cGKI is present in high concentrations (>0.1 μM) 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, and osteoclasts. The Iα isoform is found in lung, heart, dorsal root ganglia, and cerebellum. Together with the Iα isoform, the Iβ isoform is highly expressed in smooth muscle, including uterus, vessels, intestine, and trachea (14). Platelets, hippocampal neurons, and olfactory bulb neurons contain mainly the Iβ isozyme (14). The Iα and Iβ cGKs are soluble enzymes and interact with different proteins through their distinct N termini. cGKI is expressed in several brain nuclei, intestinal mucosa, kidney, adrenal cortex, chondrocytes, and lung (15–18). cGKI is anchored at the plasma membrane by myristoylation of the N-terminal Gly-2 residue. Only the membrane-bound cGKII phosphorylates cysitic fibrosis transmembrane conductance regulator (19).

Roles of cGKI

Control of Smooth Muscle Tone by cGKI—NO and other NO-generating organic nitrates stimulate the soluble guanylyl cyclase, increase cGMP levels, and thereby relax vascular and other smooth muscles. The relaxing effect of these compounds involves the activation of cGKI as shown by deleting the cGKI gene in mice (20). cGKI-deficient mice are hypertensive at 4 weeks but normotensive at an older age (20, 21). The blood pressure-lowering effect of intrarectal infusion of sodium nitroprusside is abolished in cGKI-deficient mice whereas acetylcholine infusion still lowers the blood pressure (21) suggesting that cGKI is involved in the control of vascular tone but that additional mechanisms exist. Precontracted smooth muscle strips or pressurized vessels of cGKI-deficient mice are not relaxed by acetylcholine or NO (20, 22). The defective regulation of smooth muscle tone is not confined to vascular smooth muscle but is also found in other organs. In the gastrointestinal tract, accommodation of food and generation of peristaltic waves are controlled by nonadrenergic-noncholinergic neurons, which release NO upon stimulation and relax the intestinal smooth muscle. Deletion of the cGKI gene leads to pylorus stenosis, causing severe distention of the stomach and irregular peristaltic waves that heavily retard passage of intestinal content. Deletion of cGKI did not affect cAMP-induced relaxation of vascular or intestinal smooth muscle supporting the conclusion that cAMP and cGMP use different signal pathways in smooth muscle.

Smooth muscle tone is regulated by the rise and fall of [Ca^{2+}]_{i}. Contraction is initiated by receptor-mediated generation of IP_{3} that releases Ca^{2+} from intracellular stores followed by an influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (23, 24). The rise in [Ca^{2+}]_{i} initiates contraction by activation of the Ca^{2+}/calmodulin-dependent MLCK, which phosphorylates RLC and, consequently, activates myosin ATPase. A decrease in [Ca^{2+}]_{i} inactivates MLCK and induces dephosphorylation of RLC by MLCP. Smooth muscle contraction is modulated also at constant [Ca^{2+}]_{i}, by changing the sensitivity of contraction to [Ca^{2+}] (25). cGKI interferes both with the increase in [Ca^{2+}]_{i} and with the Ca^{2+} sensitivity at several levels (Fig. 1).

Some evidence indicates that cGKI interacts with the generation of IP_{3} in smooth muscle and Chinese hamster ovary cells (26) by interfering with the inactivation of Go_{i} (27) or the activation of phosphodiesterase C (28). However, the biological significance of these findings remains unclear because a direct correlation between the cGMP-dependent phosphorylation of a target protein and the depressed IP_{3} synthesis has not been established. The IP_{3} receptor type 1 has two splice variants, the long neuronal S2" form and the peripheral short S2" form that is expressed in smooth muscle. Both splice forms are phosphorylated in vitro and in vivo at two serines by cAMP kinase and cGKI. cGKI phosphorylates preferentially Ser-1755 (29). Initial experiments suggested that phosphorylation of the IP_{3} receptor I resulted in a decreased release of Ca^{2+} from intracellular stores. Later experiments clearly showed that cAMP-dependent phosphorylation of each receptor isoform resulted in an increased Ca^{2+} release (30). cGMP-dependent phosphorylation of the peripheral IP_{3} receptor type I S2" isoform had no effect on Ca^{2+} release, whereas it increased Ca^{2+} release from the neuronal S2" isoform (30). These findings suggest that cGMP-dependent phosphorylation of the IP_{3} receptor is not relevant for smooth muscle tone regulation but may be important for neurons...
Minireview: cGMP Protein Kinases

that express cGKI but not IP$_3$ receptor-associated cGKI substrate (IRAG) (14).

The smooth muscle IP$_3$ receptor type 1 coprecipitates with cGKIβ and a 125–135-kDa protein identified as IRAG (31). IRAG is expressed together with cGKIβ in smooth muscle, platelets, and some neurons (14). It is located at the endoplasmic reticulum membrane and is preferentially phosphorylated by cGKIβ at Ser-683 and Ser-696 (bovine sequence) (32, 33). cGKIβ-dependent phosphorylation of IRAG inhibits IP$_3$-induced Ca$^{2+}$ release in intact and permeabilized cells. The IRAG sequence between amino acids 152 and 184 interacts specifically with the leucine zipper of cGKIβ (33). IRAG has a coiled-coil domain that binds in vivo with the IP$_3$ receptor type I (32). Deletion of exon 12 that codes for the N-terminal part of the coiled-coil domain results in a hypomorphic IRAG allele (32). 8-Br-cGMP-dependent relaxation of muscular and α-adrenergic receptor-induced contraction of colon and aorta muscle strips, respectively, is abolished in these mice. In agreement, 8-Br-cGMP does not attenuate noradrenaline-induced Ca$^{2+}$ transients in isolated aortic smooth muscle cells of IRAG mutants (32). However, 8-Br-cGMP-induced relaxation of K$^+$-induced smooth muscle contraction is unchanged in the IRAG mutants. In contrast, 8-Br-cGMP is unable to affect Ca$^{2+}$ release and contraction in smooth muscles from cGKI-deficient mice. These findings show for the first time that cGKI has multiple targets in vivo that contribute to smooth muscle relaxation (Fig. 1).

An additional mechanism that lowers [Ca$^{2+}$], is the direct phosphorylation of Ca$^{2+}$-activated maxi-K$^+$ (BK$_{Ca}$) channels by cGKI. Phosphorylation increased channel opening at constant [Ca$^{2+}$] (34, 35). The cGKI isoform that phosphorylates BK$_{Ca}$ channels is not known. Opening of BK$_{Ca}$ channels hyperpolarizes the membrane and closes a number of channels, including L-type calcium channels, thereby reducing Ca$^{2+}$ influx. This mechanism contributes to the regulation of vascular tone, as shown in wild type and cGKI-deficient mice (22). The cGKI-dependent regulation of BK$_{Ca}$ channels depended on specific splice variants (36) and may be mediated indirectly by cGKI-dependent activation of an associated protein phosphatase 2A (37, 38).

Regulation of BK$_{Ca}$ channels cannot account for the ability of 8-Br-cGMP to relax K$^+$-contracted smooth muscle strips of the IRAG mutant (32), because a change in BK$_{Ca}$ channel activity does not affect the membrane potential under these conditions. An alternative target for cGKI is MLCP because smooth muscle tone is decreased by dephosphorylation of the RLC (39). MLCP is a trimer comprising a 110–130-kDa regulatory MBS also identified as myosin phosphatase targeting subunit (MYPT), a 37-kDa catalytic subunit and a 20-kDa protein of unknown function (40). Several studies have shown that the inhibition of MLCP activity can be linked to increased Ca$^{2+}$ sensitivity of smooth muscle contraction. Involved in this regulatory pathway are Rho kinase, arachidonic acid, and protein kinase C and its substrate CPI-17 (40). Phosphorylation of MBS at Thr-696 (human sequence) by Rho kinase or MYPT1 kinase inhibits the activity of MLCP allowing phosphorylation of RLC and contraction at constant [Ca$^{2+}$] (25, 40). MBS is phosphorylated by cGKIα at several sites (41, 42). cGKIα is targeted specifically by its leucine zipper to MBS (41). cGKIα-dependent phosphorylation of MBS at Ser-695 inhibits the phosphorylation at Thr-696 and thereby the decrease in MLCP activity (43). This mechanism would allow a reduction in RLC phosphorylation and relaxation at constant [Ca$^{2+}$].

The individual contribution of the described cGKI-dependent mechanisms regulating smooth muscle tone might vary in different tissues. Deletion of the cGKI gene and of exon 12 of the IRAG gene suggests that phosphorylation of IRAG, BK$_{Ca}$ channels, and MBS occurs in the same cell. However, it has been shown that modulation of the Ca$^{2+}$ sensitivity is important in smooth muscles from the small intestine, whereas regulation of the [Ca$^{2+}$] is not, appears to be more important in vascular smooth muscle. In general, these findings clearly establish that NO signals through CNGKα and cGKIβ in smooth muscle and that these isozymes use different targets to affect smooth muscle tone. Regulation of Smooth Muscle Proliferation—Migration, proliferation, and dedifferentiation of vascular smooth muscle cells are considered to be essential events in the development of atherosclerosis and vascular restenosis. Many groups have reported that NO, atrio-natriuretic peptide, and membrane-permeable cGMP analogs prevent proliferation, migration, and dedifferentiation of vascular smooth muscle cells (44, 45). Recent evidence indicates that cGKI modulates gene expression through the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway either by stimulation or by its inhibition (46, 47). Differential phosphorylation of VASP might be critical in these studies. VASP, a member of the Ena/Mena/Vasp family, is an actin-binding protein that is involved in the focal adhesion and cell-to-cell contacts in many cells (48). VASP is phosphorylated by protein kinase C, cAMP kinase, and cGKI at two sites with different preference. Recently, it was reported that expression of VASP and phosphorylation of Ser-157 increased proliferation whereas phosphorylation of Ser-239 decreased proliferation (49). Surprisingly, serum as used in cell culture studies stimulates phosphorylation of Ser-157 of VASP through PKC (50), an effect of serum not noticed so far. In a mouse model of ischemic vessel growth, the presence of cGKK was essential for vessel growth (51). In a mouse model of atherosclerosis it was found (52) that smooth muscle-specific deletion of cGKK retarded the development of atherosclerotic plaques and smooth muscle proliferation in vivo. In this study (52), it was observed that stimulation of cGKK increased smooth muscle cell growth in vivo, whereas NO inhibited growth independent of cGKK. It is therefore likely that in vivo cGKK stimulates smooth muscle growth and that
the existing controversy will wane as soon as better controlled experiments are initiated.

Platelet Aggregation—In most cases, aggregation of platelets is initiated at areas where the endothelial cell layer has been destroyed. Endothelial cells release prostacyclin and NO, which prevent platelet release and aggregation. These factors raise cAMP and cGMP levels in platelets and thereby inhibit clot formation. Platelets have a high concentration of cGKIβ that is activated in response to NO and has an anti-aggregatory function (53–55). Under specific conditions, cGKI may also promote platelet activation (56), perhaps by facilitating the release of ADP (57). However, NO or cell-permeable cGMP analogs did not inhibit aggregation of cGKI-deficient platelets, whereas aggregation was prevented by cAMP-elevating agents (53). An intact platelet NO/cGKI signaling pathway is essential to prevent platelet aggregation after ischemia in vivo (53). Platelets contain two well established cGKI substrates, VASP and IRAK. Deletion of the VASP gene in mice did not grossly affect platelet aggregation (58, 59), but considerably affected the interaction of platelets with the endothelium in vivo (60). Activation of cGKI inhibited the release of Ca^{2+} from IP3-sensitive stores in wild type and VASP-deficient platelets to similar extents (58). Platelets express IRAG (14). Furthermore, platelets from the IRAG mutant mice (32, 61) have a severe defect in the cGMP-mediated prevention of aggregation, indicating that IRAG is an essential component of this pathway.

Roles of cGKII

Our knowledge about the function of cGKI is at a very preliminary state. Only three areas have been identified where cGKI plays a role: secretion, bone growth, and circadian rhythmicity.

Secretion—Intestinal Cl-/fluid secretion is increased by substances that stimulate cAMP (cholera toxin) or cGMP (guanylin, STa) levels. cGKI is highly expressed in the apical membrane of the enterocytes of the small intestine. The mucosa of cGKII-deficient mice responded normally to cAMP analogues, whereas STa-induced electrolytic anion secretion was blocked. Active cGKI phosphorylated cystic fibrosis transmembrane conductance regulator and increased Cl− and water secretion. These results established that cGKI is essential for guanylin/STa-dependent secretion in the small intestine (62, 63).

NO/cGMP were reported to modulate calcium release in the kidney. Deletion of the cGKI gene had no effect on renin release in isolated kidneys or kidney cells, whereas the inhibitory effect of NO on renin release was blunted in cGKI-deficient mice (64). Blood pressure was not affected in cGKI+/− mice. This is interesting because it was reported that activation of cGKI increased the secretion of aldosterone in rat adrenal cortical cells (65). It is possible that the opposite effect of cGKI on two blood pressure-elevating factors cancelled each other in the knock-out animals.

Bone Growth—The particulate Gcs, GC-A and GC-B, are expressed abundantly in mouse tibial epiphysis and vertebrae (66). Cultivation of mouse tibias in the presence of 1 mM brain-natriuretic peptide, a ligand for GC-A and GC-B, induced an increase in total bone length. Transgenic mice overexpressing BNP exhibited skeletal overgrowth which was restricted to those bones that grow by endochondral ossification. cGKI and -II are expressed in the growth zone of bones (62). The deletion of cGKI has no apparent effect on the growth of the skeleton (20). In contrast, cGKII-deficient mice are dwarfs with 16–30% shorter limbs. cGKI is essential for the CNP/cGMP-mediated endochondral ossification (67) and regulates autonomous bone growth.

Circadian Rhythmicity—The suprachiasmatic nucleus (SCN) harbors the circadian clock pacemaker that runs on a close to 24-h time scale and is reset to the external time period by multiple pathways (68). Both cGks are expressed in the SCN. Studies with cGKI−/− mice showed that cGKI influenced the phase shift of the circadian clock at the onset of the wheel running activity (69). The involvement of cGKI in the regulation of the circadian clock was confirmed (70) in the isolated rat SCN, although in that system cGKI was required for night to day progression of the clock (71).

Behavior and cGks

cGks and the Mammalian Brain—Although cGKI is expressed in many neurons, deletion of the gene resulted only in mild neurologic effects such as a moderate enhanced anxiety-like behavior and a hyposensitivity to acute alcohol intake (18). Although the neural expression of cGKI is rather restricted, a number of important phenotypes are associated with tissue-specific deletion of cGKI (for an extensive discussion see Ref. 72). cGKI expressed in sensory neurons of dorsal root ganglia is necessary for the correct guidance of sensory axons during embryonic development (73). Recent evidence suggests that cGIKs is also involved in some forms of pain perception (74). cGKIβ is involved in hippocampal long term potentiation, a paradigm for learning, as shown in cell culture (75) and in hippocampus-specific cGKI knock-out mice (76). The biological significance of this finding is unclear because the cGKI knock-outs showed no learning defect in several tests (76). The target(s) of cGKI in hippocampal and dorsal root neurons are unknown.

Cerebellar Purkinje neurons express high levels of cGKIs and the G-substrate, a peptide specifically phosphorylated by cGKI. The phosphorylated G-protein is an inhibitor of protein phosphatase 1/2A (77, 78). It has been hypothesized that inhibition of the protein phosphatasea allows phosphorylation of the AMPA receptor by other kinases and its internalization leading to long term depression. Indeed, coincidence of an increase in [Ca^{2+}] and cGKI activity is necessary for induction of long term depression (79) and cerebellar learning (80).

Food-searching Behavior and cGks—cGks were also identified in a large number of invertebrates. Drosophila melanogaster has two cGK genes, dg1 and dg2 (81). dg2 encodes a protein kinase that is related to the mammalian cGKI gene (1, 82). Analysis of naturally occurring Drosophila variants in food-searching behavior indicated that a high activity of the dg2 kinase is a major determinant of versus sitter behavior (83). A similar situation has been identified in honey bees. A cGKI-like kinase activity is up-regulated when the young bees change from hive work to foraging (84). The opposite change has been found in Caenorhabditis elegans. In these animals, long distance roaming for food is associated with a decreased cGKI activity (85). Interestingly, the cGKI gene involved in this behavior is more related to mammalian cGKI than to cGKI (86) pointing to the possibility that increased cGKI activity is related to a sedentary life style in vertebrates. The above studies unequivocally demonstrate that cGks are involved not only in cardiovascular physiology but also regulate complex central nervous processes and that even subtle changes in cGKI activity can lead to naturally occurring behavioral variants.

Acknowledgment—I thank Robert Feil for reading the manuscript.

REFERENCES

1. Pfeifer, A., Ruth, P., Dostmann, W., Sausbier, M., Klett, P., and Hofmann, F. (1999) Rev. Physiol. Biochem. Pharmacol. 135, 105–149
2. Francis, S. H., and Corbin, J. D. (1999) Crit. Rev. Clin. Lab. Sci. 36, 275–328
3. Ruth, P., Pfeifer, A., Kamm, S., Klett, P., Dostmann, W. R., and Hofmann, F. (1997) J. Biol. Chem. 272, 10522–10528
4. Gamm, D. M., Francis, S. H., Angelotti, T. P., Corbin, J. D., and Uhler, M. D. (1995) J. Biol. Chem. 270, 27390–27398
5. Landgraf, W., Hofmann, F., Felton, J. T., and Huggins, J. P. (1990) Biochemistry 29, 9921–9929
6. Zhao, J., Trewhella, J., Corbin, J., Francis, S., Mitchell, R., Brushia, R., and Walsh, D. (1997) J. Biol. Chem. 272, 31929–31936
7. Wall, M. E., Francis, S. H., Corbin, J. D., Grimes, K., Richie-Jannetta, R., Kotera, J., Macdonald, B. A., Gibson, R. R., and Trewhella, J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2380–2385
8. Smith, J. A., Francis, S. H., Walsh, K. A., Kumar, S., and Corbin, J. D. (1996) J. Biol. Chem. 271, 20756–20762
9. Wyatt, T. A., Lincoln, T. M., and Fryewazsky, K. B. (1991) J. Biol. Chem. 266, 21274–21280
10. Vaandrager, A. B., Hogema, B. M., Edixhoven, M., van den Burg, C. M., Bot, A. G., Klett, P., Ruth, P., Hofmann, F., Van Damme, J., Vandekerckhove, J., and de Jonge, H. R. (2003) J. Biol. Chem. 278, 28651–28658
11. Francis, S. H., Potet-Smith, C., Busch, J. L., Richie-Jannetta, R., and Corbin, J. D. (2002) Front. Biosci. 7, d580–d592
12. Hofmann, F., Genshemer, H. P., and Gobel, C. (1985) Eur. J. Biochem. 147, 361–365
13. Smith, J. A., Reed, R. B., Francis, S. H., Grimes, K., and Corbin, J. D. (2000) J. Biol. Chem. 275, 154–158
14. Geiselhoringer, A., Gaisa, M., Haufmann, F., and Schlossmann, J. (2004) FEBS Lett. 575, 19–22
15. el-Husseini, A. E., Bladen, C., and Vincent, S. R. (1995) J. Biol. Chem. 270, 361–365
16. M. Antl, F. Hofmann, and J. Schlossmann, personal communication.
