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Function of cGMP-Dependent Protein Kinases as Revealed by Gene Deletion

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Hofmann, F., R. Feil, T. Kleppisch, and J. Schlossmann. Function of cGMP-Dependent Protein Kinases as Revealed by Gene Deletion. *Physiol Rev* 86: 1–23, 2006; doi:10.1152/physrev.00015.2005.—Over the past few years, a wealth of biochemical and functional data have been gathered on mammalian cGMP-dependent protein kinases (cGKs). In mammals, three different kinases are encoded by two genes. Mutant and chimeric cGK proteins generated by molecular biology techniques yielded important biochemical knowledge, such as the function of the NH_2-terminal domains of cGKI and cGKII, the identity of the cGMP-binding sites of cGKI, and the substrate specificity of the enzymes. Genetic approaches have proven especially useful for the analysis of the biological functions of cGKs. Recently, some of the in vivo targets and mechanisms leading to changes in neuronal adaptation, smooth muscle relaxation and growth, intestinal water secretion, bone growth, renin secretion, and other important functions have been identified. These data show that cGKs are signaling molecules involved in many biological functions.

I. INTRODUCTION

Nitric oxide (NO)-generating drugs (e.g., glyceryl trinitrate or sodium nitroprusside) have been used to treat cardiovascular diseases in humans for more than 100 years. In 1977, Murad's group (126) reported that these compounds activate the guanylyl cyclase in various tissues. Three years later, Furchgott and Zawadzki (80) reported that acetylcholine relaxed blood vessels by a factor generated in the endothelium (EDRF). Seven years later, EDRF was identified as the gas NO (117, 207). Since then, a plethora of studies with NO-donor compounds and NO synthase (NOS) inhibitors underscored the importance of NO for the function of numerous tissues including the nervous, cardiovascular, gastrointestinal, endocrine, and immune systems. NO is generated by three

different isozymes, and in many cells, NO increases the concentration of cGMP by activation of the soluble guanylyl cyclase (sGC) (77, 126). cGMP is also generated by membrane-bound particulate guanylyl cyclases (pGCs, e.g., GC-A, GC-B, and GC-C) (84). GC-A and GC-B are major receptors for a family of natriuretic peptides released from the heart and vascular endothelium, like atrial 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 (84). Further analysis of the cGMP system identified a number of intracellular targets for cGMP. For example, cGMP binds to cAMP-specific phosphodiesterases (PDEs) and, thereby, modulates the concentration of cAMP (236). 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 systems (17, 106). Most cells contain at least one of three cGMP-dependent protein kinases (cGKs): cGKIα, cGKIβ, or cGKII (74, 105, 162, 166, 213) that are targeted by their amino termini to distinct substrates and are involved in the regulation of different cellular functions.

Several effects of NO are mediated independent of the cGMP/cGK signaling pathway (102). The same caution is necessary for the analysis of cGMP effects, because *1*) cGMP has several effectors that may be used simultaneously 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 (27, 175). We have tried to avoid the interpretation of ambiguous results. This review mainly concentrates on results obtained by total or tissue-specific deletion of the cGK genes and of some of their substrates.

II. BIOCHEMICAL FACTS ON cGMP-DEPENDENT PROTEIN KINASES

A. Genes and Isozymes

The 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* (74, 213). Mammals have two cGK genes, *prkg1* and *prkg2*, that encode cGKI and cGKII. The NH_2 terminus (the first 90–100 residues) of cGKI is encoded by two alternative exons that produce the isoforms cGKIα and cGKIβ. The enzymes have a rodlike structure and are activated at submicromolar to micromolar concentrations of cGMP (83, 233). They are composed of three functional domains: an NH_2-terminal domain, a regulatory domain, and a catalytic domain (for details, see Refs. 74, 213). The regulatory domain contains two tandem cGMP-binding sites that bind cGMP with high and low affinity and interact allosterically. Occupation of both binding sites induces a large change in secondary structure (149) to yield a more elongated molecule (286, 309). The catalytic domain contains the MgATP- and peptide-binding pockets. Binding of cGMP to both sites in the regulatory domain releases the inhibition of the catalytic center by the NH_2-terminal autoinhibitory/pseudosubstrate site and allows the phosphorylation of serine/threonine residues in target proteins and in the amino-terminal autophosphorylation site. Activation of heterophosphorylation may be preceded by autophosphorylation, which increases the basal activity of cGKI and cGKII (75, 255, 278, 301) and is initiated by the binding of low cGMP concentrations to the high-affinity site of cGKI (108, 256). In addition to controlling activation and inhibition of the catalytic center, the NH_2 terminus serves two other functions: *1*) dimerization, cGKs are homodimers that are held together by a leucine zipper present in the NH_2 terminus; and *2*) targeting, the enzymes are targeted to different subcellular localizations by their NH_2 termini.

B. Tissue Distribution

cGKI is present at high concentrations in all smooth muscles, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular junction end plate, and kidney. Low levels have been identified in cardiac muscle, vascular endothelium, granulocytes, chondrocytes, osteoclasts, and diverse brain nuclei (69, 127). The Iα isozyme is found in lung, heart, dorsal root ganglia (DRG), and cerebellum. Together with the Iα isozyme, the Iβ isozyme is highly expressed in smooth muscle, including uterus, vessels, intestine and trachea (86). Platelets, hippocampal neurons, and olfactory bulb neurons contain mainly the Iβ isozyme (86). The Iα and Iβ cGKs are soluble enzymes and interact with different proteins through their distinct NH_2 termini. cGKII is expressed in several brain nuclei, intestinal mucosa, kidney, adrenal cortex, chondrocytes, and lung (50, 59, 166, 292). cGKII is anchored at the plasma membrane by myristoylation of the NH_2-terminal Gly2 residue. Only the membrane-bound cGKII phosphorylates the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) (279).

C. Substrates

Over 10 substrates have been identified that are phosphorylated in vivo by cGKI and one that is modified by cGKII (see Table 1 for details). Additional proteins were identified as substrates in expression systems (Table 1),

TABLE 1. *cGK substrates*

Substrate	Molecular Weight, × 1,000	cGK Isoform	Tissue or Cells	Function of Phosphorylation	Reference Nos.
Established cGK substrates (with probable function of phosphorylation)					
BK_{Ca}	130	cGKI	Smooth muscle	Increased open probability; membrane hyperpolarization	241
G substrate	32	cGKI	Cerebellum	Protein phosphatase inhibitor; initiation of LTD	62
IP_3 receptor type I	230	cGKI	Cerebellum	Stimulation of calcium release from IP_3-sensitive stores	97, 284
IRAG	125	cGKIβ	Smooth muscle, platelets	Reduced calcium release from IP_3-sensitive stores	87, 244
MYPT1	130	cGKIα	Smooth muscle	Inhibition of myosin phosphatase inhibition by rho kinase; decreased calcium sensitization	300
PDE5	100	cGKI	Smooth muscle, platelets	Enhanced cGMP degradation	235
Phospholamban	6	cGKI	Vascular smooth muscle	Enhanced calcium uptake by the calcium ATPase SERCA	148
RGS2	24	cGKIα	Smooth muscle	Inhibition of IP_3 generation	270
Sox9	56	cGKII	Chondrocytes	Bone development	39
Telokin	17	cGKI	Smooth muscle	Inhibition of MLCK activity	285
VASP	46/50	cGKI	Smooth muscle, platelets, hippocampus	Regulation of the actin cytoskeleton, vesicle trafficking	28, 98
Potential cGK substrates (phosphorylation in heterologous systems or with unidentified function of phosphorylation)					
CFTR	200	cGKII	IEC-CF7 cells (intestinal cell line)	Stimulation of chloride channel	279
CRP2	23	cGKI	Smooth muscle, enteric neurons	Regulation of smooth muscle tone	114, 242
Hsp27	27	cGKI	Platelets	Decrease of actin polymerization in vitro	29
Septin-3	40	cGKI	Brain	Vesicle trafficking	303
Rap1GAP2	90	cGKI	Platelets	Inhibition of Rap1	249
RhoA	22	cGKI	Smooth muscle, hippocampus	Reduced MLC phosphorylation, vesicle trafficking	61
TRPC3	97	cGK1	HEK293	Inhibition of store-operated calcium influx	145
TxA_2 receptor (TP) 1α	40	cGK1	HEK293	Desensitization of TP 1α signaling	225

See text for definitions.

but their phosphorylation has not been shown to occur in an intact native cell or organism. The majority of identified substrates are components of other signaling pathways, such as ion channels, G proteins, and associated regulators or cytoskeleton-associated proteins. Details on the function of these proteins are given in the following sections. The substrate specificity of cGKs depends on the distinct NH_2 terminus of each isozyme, e.g., the inositol 1,4,5-trisphosphate (IP_3) receptor-associated cGKI substrate (IRAG) interacts only with the NH_2 terminus of the Iβ isozyme (244), whereas the myosin phosphatase targeting subunit (MYPT1) interacts specifically with the NH_2 terminus of the Iα isozyme (268). Inhibition of IP_3 synthesis depended specifically on the expression of the Iα isozyme (234, 270) and could not be achieved by expression of the Iβ isozyme (182). These two isozymes differ only in their NH_2 terminus. Interaction with CFTR is specific for the NH_2 terminus of the cGKII isozyme (279). The isozyme specificity of other substrates has not yet been investigated.

II. cGMP-DEPENDENT PROTEIN KINASE SIGNALING IN THE NERVOUS SYSTEM

NO regulates a broad range of functions in the nervous system. Deletion of the genes coding for different isoforms of NOS in mice causes prominent alterations in neuronal development, synaptic plasticity and learning, behavior, and nociception (67, 106). On the basis of pharmacological studies, cGKs have been linked to a number of the above functions. Compelling evidence regarding the role of specific isoforms was missing until the recent analysis of knockout mice that chronically lack cGKI or cGKII either in all cells (conventional knockout) or in a specific subset of neurons (conditional knockout). The interpretation of studies with conventional knockout mice (130, 211, 212, 292) is limited by the multiple phenotypes of these mutants. For example, the reduced life span of cGKI null mutants precludes studies of synaptic plasticity and behavior in adult animals. With the use of the Cre/lox system (185), the cGKI gene was deleted

selectively in the hippocampus (131) and in cerebellar Purkinje cells (66), yielding fully viable mice. Neuronal phenotypes of cGK-deficient mice are summarized in Table 2.

A. Axon Guidance

The precise and selective formation of the myriad connections between neurons during development is essential for the function of the vertebrate nervous system. It relies on the correct pathfinding and target recognition by growth cones. The latter is controlled by attractive and repulsive cues in the extracellular environment, e.g., ephrins, netrins, slits, and semaphorins, that act through specific cell surface receptors and intracellular signaling cascades (for review, see Refs. 51, 273). Cyclic nucleotides are believed to be important regulators of the correct wiring of neurons based on the following findings: *1*) increasing levels of cGMP transform the action of semaphorin 3A (Sema3A) on axonal growth in *Xenopus* spinal neurons from repulsion to attraction and protect rat sensory growth cones from Sema3A-induced collapse (261), and *2*) guanylyl cyclase-mediated cGMP synthesis is an important element of the signaling cascades initiated by semaphorin 1A and Sema3A in *Drosophila* motor axons (11) and mammalian cortical apical dendrites (217). However, mice lacking the neuronal NOS showed only a reduction of the number of bifurcations in the dendritic tree of motor neurons without a generalized defect in guidance of nerve axons (118). Thus the nature of cGMP-dependent effects on axon guidance and their relevance in vivo remained largely a matter of speculation until mice lacking cGK proteins were available for analysis.

cGKIα is expressed in sensory axons of the DRG, while the majority of spinal cord neurons lacks cGKI (221, 246). Furthermore, its localization in the filo- and lamellipodia of growth cones in cultured DRG neurons is well-suited for transduction of signals in growing sensory axons during development (246). Normally, once entering the dorsal root entry zone of the developing spinal cord, sensory axons bifurcate into a rostral and caudal branch extending over several segments without growing into the gray matter. The analysis of the trajectories of sensory axons in the spinal cord of mouse embryos revealed that

TABLE 2. *Neuronal and cardiovascular phenotypes of conventional and conditional cGK knockout mice*

Deleted Gene	Type of Knockout	Phenotype	Reference Nos.
prkg1 (cGKI)	Null mutation	Defective axon guidance in the dorsal root entry zone and impairment of nociceptive flexion reflexes	246
		Reduced inflammatory hyperalgesia without alterations in acute thermal nociception	271
		Impaired NO/cGMP-dependent vasorelaxation	134, 212, 241
		Impaired ischemia-induced angiogenesis	304
		Enhanced platelet activation during ischemia/reperfusion	178
		Blunted cardiac negative inotropic response to cGMP, but normal negative inotropic response to carbachol	291
prkg1 (cGKI)	Hippocampus specific (pyramidal cells)	Impairment of protein synthesis-dependent LTP in adult mice without overt effect on spatial learning	131
	Cerebellum specific (Purkinje cells)	Impairment of cerebellar LTD and motor learning	66
	Smooth muscle specific	Reduced development of SMC-derived plaque cells and atherosclerosis on ApoE$^{-/-}$ background	297
		Normal development of restenosis in response to carotid injury	171
	Cardiomyocyte specific	Blunted cardiac negative inotropic response to cGMP, but normal negative inotropic response to carbachol	291
		Normal cardiac hypertropy in response to pressure overload	68
prkg2 (cGKII)	Null mutation	Loss of STa and cGMP-stimulated intestinal water secretion, loss of cGMP stimulated short-circuit current, reduced bone length, and reduced endochondreal ossification	211
		Loss of cGMP-mediated inhibition of renin expression and secretion in renal juxtaglomerular cells	283
		Altered light-induced resetting of the circadian clock during early night	204
		Enhanced anxiety, elevated ethanol consumption combined with hyposensitivity to hypnotic doses of the drug	292

axons lacking cGKI extended predominantly into a single direction (246). This branching defect resulted in *1*) a reduced number of sensory axons in the spinal cord of newborn mutants and *2*) a substantial impairment of the nociceptive flexion reflexes compared with their wild-type littermates (246). These findings were the first in vivo proof that cGKI is required for the correct guidance and connectivity of axons originating from DRG sensory neurons.

A further validation is needed for a function of cGMP/cGKI signaling in neuronal path finding within the mammalian brain (187, 217). In vitro experiments with pharmacological agents suggested that generation of cGMP and activation of cGKI are involved in Sema3A-induced growth cone collapse (54). In contrast to this report, cultured DRG neurons from wild-type mice and mice lacking cGKI showed no difference in the degree of the Sema3A-induced collapse (246). Moreover, treating wild-type neurons with cGMP led to a reduction of growth cone collapse in the presence of Sema3A, and this effect was absent in cGKI-deficient neurons. These findings not only challenge the view that cGKI represents an essential component of the Sema3A-induced pathway leading to growth cone collapse, but rather indicate an inhibitory function of cGKI. So far, the targets transducing the effect of cGKI on axonal growth and growth cone collapse are unknown. The cGMP/cGKI pathway could change the growth cone direction by triggering the remodeling of the actin cytoskeleton, e.g., by phosphorylation of the actin-organizing protein VASP (150).

B. Synaptic Plasticity and Learning

Long-term potentiation (LTP) and long-term depression (LTD) of synaptic activity can be induced by specific activity patterns in glutamatergic synapses in various brain regions. Such activity-dependent changes of the synaptic transmission represent potential cellular mechanisms for learning and memory formation. Thus hippocampal LTP is believed to be associated with spatial learning (for review, see Ref. 36), and cerebellar LTD with motor learning (24). LTP-like changes of synaptic transmission in the amygdala may underlie fear conditioning (230), and hippocampal LTD may be a prerequisite for novelty acquisition (174). We will focus on those forms of synaptic plasticity that have been demonstrated to be, at least partially, mediated through NO/cGMP-dependent mechanisms (67, 106).

1. *LTP in the hippocampal CA1 region and spatial learning*

NO, generated mainly by Ca^{2+}/calmodulin-regulated eNOS, is thought to act as a retrograde messenger during the induction of LTP in the hippocampal CA1 region (8, 19, 199). Accordingly, mice lacking either endothelial NOS (eNOS) or both eNOS and neuronal NOS (nNOS) display a marked reduction in hippocampal LTP (260, 294), while LTP is normal in nNOS-deficient mice (199). There is also evidence supporting the view that NO mediates its effect in the presynaptic neuron via activation of sGC, an increase of the cytosolic cGMP concentration, and activation of cGK (8, 9, 22, 312). Zhuo and co-workers (312) observed an enhancement of LTP following a weak tetanic stimulation in the presence of cGK activators, and, conversely, a suppression of LTP in the presence of cGK inhibitors. In conflict with their findings, hippocampal LTP was normal in conventional knockout mice lacking cGKI, cGKII, or both genes (double knockout mice) (130).

Null mutants used in this study lack cGKs in all cells during their entire ontogenesis. Hippocampal neurons express predominantly cGKIβ (69, 86, 131). cGKI has been implicated in the LTP elicited in cultured hippocampal pyramidal cells (8). In agreement with the data in cGKI null mutants, a mouse line with a hippocampus-specific cGKI knockout ($cGKI^{hko}$) (131) that lacked cardiovascular and gastrointestinal defects associated with the null mutation showed normal basal synaptic transmission and normal hippocampal LTP within the first hour after a single tetanus (131). This result further emphasized that cGKI does not serve a critical function in the early phase of LTP (E-LTP). A protein synthesis-dependent late phase of LTP (L-LTP) in the CA1 region can be induced by multiple strong tetanic stimulation of Schaffer collaterals (12, 76) and may partially depend on the generation of NO and cGMP (169, 170). Supporting the view that cGKI serves as a possible distal target for NO/cGMP in L-LTP, adult $cGKI^{hko}$ mice (12–14 wk of age) displayed an impairment of hippocampal LTP following multiple episodes of strong theta burst stimulation. Anisomycin, a protein synthesis inhibitor, reduced L-LTP in adult control mice to a level similar to that observed in the $cGKI^{hko}$ mice in the absence of anisomycin, but had no effect on LTP in $cGKI^{hko}$ mice (131). Thus cGKI-dependent LTP in response to multiple episodes of strong theta burst stimulation meets a hallmark of L-LTP, the dependence on protein synthesis. NO/cGMP-dependent L-LTP has been shown to require activation of the transcription regulator CREB (169, 170). The potential functional role of cGKI for L-LTP was supported recently by experiments that showed that cGKI is located presynaptically and postsynaptically in cultured hippocampal neurons (288). cGKI increased both presynaptically and postsynaptically the membrane localization of synaptophysin labeled puncta that contained the GluR1 protein (288). A further peculiarity of the cGKI supported L-LTP was that it was absent in juvenile (3–4 wk of age) cGKI mutants, no matter whether the cGKI deficiency was global or hippocampus specific (131). These findings suggest that a cGKI-depen-

dent component of L-LTP develops during maturation to adulthood.

The function of cGKI-mediated L-LTP for hippocampus-dependent learning was tested in a discriminatory water maze task (10) and in contextual fear-conditioning (232). The deficiency of hippocampal cGKI had no impact on the performance of adult $cGKI^{hko}$ mice in either test (131). These findings do not rule out categorically that cGKI has a function in learning and memory. For example, hippocampus-specific AMPA receptor GluR1 knockout mice also exhibit impaired LTP and normal spatial reference memory (water maze task), but they have a defect in spatial working memory (spontaneous alteration task) (226, 307).

2. Cerebellar LTD and motor learning

The inhibitory output of the cerebellar cortex to vestibular and cerebellar nuclei is derived solely from Purkinje cells (PCs) and is thought to serve important functions in motor coordination and reflexive behaviors, and learned adaptations of these behaviors. PCs themselves receive excitatory inputs from *1*) a large number of parallel fibers (PFs) originating from granule cells within the granular layer and *2*) a single climbing fiber (CF) originating from the inferior olive (for review, see Ref. 119). LTD of the glutamatergic synaptic transmission from PFs onto PCs can be induced by specific activity patterns, e.g., when PFs are repeatedly activated in conjunction with the climbing fiber converging onto the same PC. As discussed for LTP in the hippocampus, LTD might represent a cellular correlate for learning behavior associated with the cerebellum, e.g., various simple forms of motor learning, such as adaptation of ocular movements and eyeblink conditioning (24, 32, 119, 181, 224). Numerous findings support the concept that NO released presynaptically plays a critical role in cerebellar LTD and that it acts at the postsynaptic site through activation of a cGMP-dependent signaling mechanism (for review, see Ref. 119). For example, LTD is blocked by inhibitors of NOS and sGC (23), and it is abolished in nNOS mutant mice (155). On the other hand, NO or cGMP can induce LTD when present coincidently with a rise in postsynaptic Ca^{2+} (153, 154). Interestingly, PCs contain fairly high levels of cGKIα (109, 167). Initially, a role of cGK in cerebellar LTD was suggested from studies using enzyme inhibitors with variable specificity (95, 153). The ultimate proof was provided by a genetic approach using mice with a PC-specific disruption of the cGKI gene ($cGKI^{pko}$ mice) (66). These conditional mutants lacked generalized abnormalities in the cerebellum, i.e., they showed a normal architecture and cellular morphology of the cerebellar cortex, normal CF innervation of PCs, and normal Ca^{2+} signals in response to CF stimulation (66). Disruption of the cGKI gene in PCs caused a nearly complete loss of cerebellar LTD (66). This finding unequivocally identifies cGKI as a critical component in the signaling pathway underlying the induction of LTD at PF-PC synapses (see Fig. 1).

In line with the findings in nNOS-deficient mice, $cGKI^{pko}$ mice showed normal general motor performance as assessed by footprint patterns, the runway, and rotarod

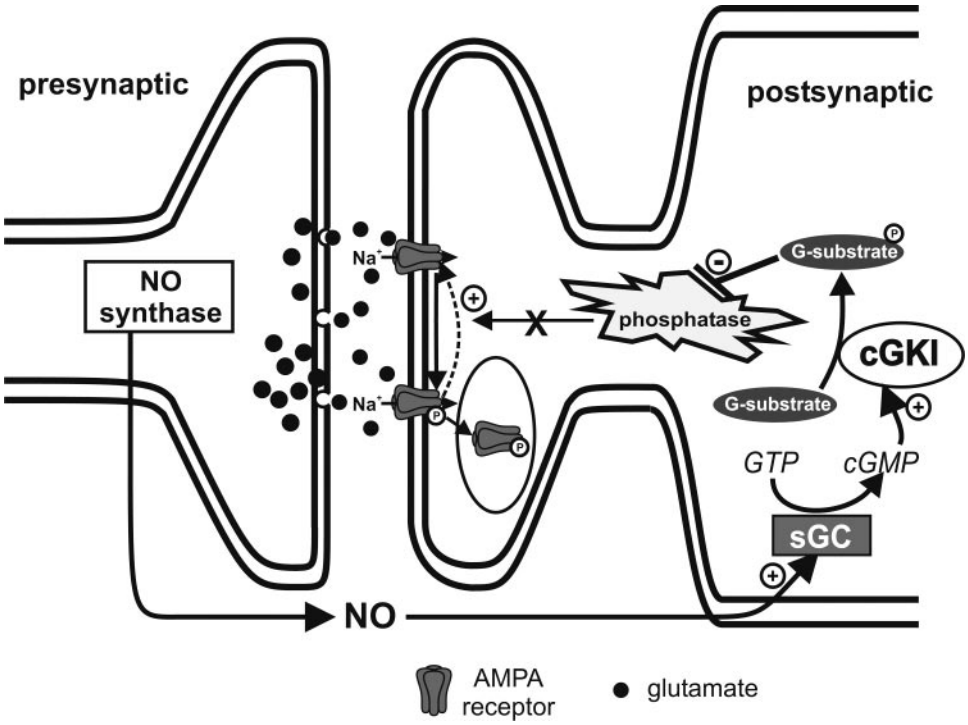

FIG. 1. Schema of the possible contribution of cGMP kinase I (cGKI) to cerebellar long-term depression (LTD) induction. Presynaptic stimulation of parallel fibers releases nitric oxide (NO) and glutamate. Glutamate activates postsynaptic AMPA receptors and increases Na^+ influx and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). NO is an anterograde messenger that stimualtes cGMP synthesis and cGKI activity in the postsynaptic Purkinje cells. cGKI phosphorylates the G-substrate, an inhibitor of protein phosphatase I/II. This leads to increased phosphorylation of the AMPA receptor, and its internalization decreases the depolarizing effect of glutamate release.

tests (66). PCs represent the convergence point for inputs from the vestibular organ (via mossy fiber and PF pathway) and the retinas (via CF pathway). On the basis of this intriguing feature, cerebellar LTD has been proposed to be involved in the adaptation of the vestibulo-ocular reflex (VOR), which is important for stabilizing images on the retina during head movements. Indeed, $cGKI^{pko}$ mice exhibited a marked defect in the adaptation of the VOR (66), similarly as described earlier for nNOS mutants (125).

The exact mechanisms how cGKI in PCs regulates LTD and cerebellum-dependent learning is unclear. According to a generally accepted view, the extent of LTD depends on the levels of internalization of the phosphorylated AMPA receptor (41, 120, 289). Phosphorylation of the AMPA receptor and its internalization is enhanced by inhibition of the protein phosphatases 1/2A (2). Intriguingly, protein phosphatase 1/2A can be inhibited by the phosphorylated form of the well-characterized G-substrate, which is highly expressed in PCs (93). Therefore, a likely signaling pathway is as follows: NO/cGMP-dependent activation of cGKI results in phosphorylation of the G-substrate in PCs, followed by inhibition of protein phosphatases 1/2A resulting in an increased phosphorylation and endocytosis of the AMPA receptor. The removal of the AMPA receptor from the postsynaptic side induces LTD associated with improved motor learning.

C. Behavior

NO is known to affect various forms of behavior in rodents (for review, see Ref. 106). For example, inhibition of nNOS or deletion of the corresponding gene caused a strong enhancement of aggressiveness, distorted sexual behavior, and a decrease in anxiety-like behavior in male animals (47, 194, 282, 306). Endothelial NOS appears to serve the opposite function in aggressive behavior. Male mice lacking this isoform display fewer attacks and a largely increased latency to attack the stimulus male in the resident-intruder paradigm in relation to wild-type mice (48). On the basis of the correlation of ethanol consumption with aggressive (72, 121, 188, 214) and anxiety-like behavior (100), it has further been suggested that NO signaling might control ethanol drinking behavior. Treatment with NOS inhibitors reduced alcohol consumption in several rat strains tested under different conditions (30, 147, 228). On the other hand, activation of the NO pathway opposes effects of acute ethanol administration (70). These findings indicated that NO may actually activate a feedback inhibitory loop following exposure to ethanol. In line with this idea, nNOS-deficient mice show increased ethanol consumption (263). The cGKII isozyme that is widely distributed throughout the mammalian brain (59, 67, 292) has been recently demonstrated to be involved in the control of anxiety-like behavior and the behavioral effects of ethanol (292). Although cGKII-deficient and wild-type mice showed no difference in the resident/intruder paradigm assessing aggressive behavior, cGKII mutants exhibited strongly increased anxiety. The anxiety-related behavior was not due to changes in locomotor activity, which was normal in mice lacking the cGKII. Following intraperitoneal injection of ethanol, the loss of righting reflex occurred with the same time course in wild-type and cGKII null mutants. However, the two genotypes differed significantly in the latency to regain the righting reflex, i.e., the measure for the persistence of the hypnotic effect of ethanol. This parameter was significantly reduced in two $cGKII^{-/-}$ mouse lines with a different genetic background. Importantly, there was no difference in ethanol metabolism between cGKII mutants and the wild-type mice. The two genotypes also showed marked differences in their ethanol consumption in a free choice test without a general difference in place preference (292). In rodents, ethanol consumption partially depends on its sweet-bitter flavor, i.e., the sweet taste component can enhance ethanol preference (263). However, $cGKII^{-/-}$ mice showed no difference from the wild-type in drinking tests with solutions containing 4% sucrose or 0.06% saccharin. Thus cGKII knockout mice showed an increased anxiety-like behavior, a reduced sensitivity to the hypnotic effect of ethanol, and an enhanced voluntary consumption of ethanol.

D. Circadian Rhythmicity

Many behavioral responses of animals (e.g., feeding, drinking, and locomotor activity) and the underlying neurohumoral activities are organized in circadian rhythms. These rhythms are driven and adaptively synchronized to the solar light cycle by internal clocks. More than 20 years ago, the master clock mechanism controlling circadian patterns in mammals was discovered within a tiny structure at the base of the hypothalamus, the suprachiasmatic nucleus (SCN) (189, 265). The generation and maintenance of mammalian circadian rhythmicity relies on complex interlocked transcriptional/translational feedback loops involving a whole set of clock genes including the period genes, *mPer1* and *mPer2* (3, 88). SCN neurons express mPer transcripts in a circadian fashion. As the corresponding periods are not exactly 24 h, the clock needs to be synchronized to the dark-light cycle, e.g., by inputs to the SCN via the retinohypothalamic tract (190). Light perceived during the early night elicits phase delays, while light perceived during the late night causes phase advances.

An important and conserved feature of the nocturnal phase is its regulation through NO/cGMP-mediated signaling mechanisms (63, 220). For example, light-induced

phase shifts have been shown to involve glutamatergic activation of NMDA receptors leading to Ca^{2+} influx into SCN neurons, NOS activation, and generation of NO (52, 53, 163). In concert with the nocturnal sensitivity of the SCN to light, glutamate, and NO, the activity of neurons in the SCN appears to be sensitive to cGMP analogs (220) and to cholinergic regulation via muscarinic receptors (18, 164). Muscarinic activation has been suggested to represent a physiological stimulus leading to increased cGMP levels and cGK activity in the SCN after midnight (163, 164, 275). Application of cGK inhibitors at the end of the subjective night delayed the neuronal activity rhythm of SCN slice preparations and the wheel-running activity rhythm in the rat (180, 275, 290). Moreover, within the corresponding time window, cGK itself appears to regulate intrinsic oscillations of the cGMP level and mPer1 expression in the SCN (275). Apparently none of these parameters depends on cGK activity during subjective day time.

Both cGKI and cGKII have been reported to be expressed in subpopulations of SCN neurons (60, 69, 204, 227). Mice with an inactivation of the 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 (204). Disruption of the cGKII gene, however, resulted in an impaired light-induced entrainment of the circadian clock within a definite time window. Compared with 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 (204). 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. These findings indicate that cGMP signaling mediated by cGKII is not required for normal clock function. However, cGKII appears to be an important effector of cGMP in the clock-resetting mechanism during early night, presumably by regulating clock gene expression.

In contrast to the results obtained with cGKII-deficient mice, pharmacological studies have placed the cGKs into the signaling pathway for phase advances (180, 290), and a recent in vitro study reported an essential role of cGKII in the progression of the circadian cycle (274). The reason for these discrepancies is 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. A function of cGKI, which is also expressed in the SCN (227), is unlikely, since conditional knockout mice lacking cGKI in the SCN did not show a significant defect in circadian rhythmicity.

E. Nociception

Efforts to elucidate the role of NO and cGMP in nociception yielded somewhat puzzling results: both pro- and antinociceptive effects of NO and cGMP have been observed in behavioral studies (195, 262, 272, 281). These findings may be due to the existence of multiple NOS isoforms. For example, mice with a deletion of the gene coding for inducible NOS showed reduced thermal hyperalgesia following zymosan injection and lacked the spinal prostaglandin synthesis that is typically induced by peripheral nociceptive stimuli, while treatment with the NO donor RE-2047 restored normal responses (92). On the other hand, mice lacking nNOS showed normal Formalin-induced nociceptive licking behavior (46). Moreover, nNOS-derived NO has been suggested to attenuate the acute analgesic actions of morphine. In a hot plate test, the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) facilitated morphine analgesia in wild-type but not nNOS-deficient mice (157). The diverse effects of NO/cGMP on nociception may also reflect the existence of different subsets of primary sensory neurons or the fact that the two messengers modulate pain processing via multiple cellular and molecular mechanisms. Despite these ambiguities, evidence is mounting that a pathway via cGMP and cGKI influences the processing of nociceptive information at the level of the spinal cord. The cGKIα is highly expressed in neurons of the dorsal root ganglion neurons, specifically in axonal processes in laminae I and II of the spinal cord, where it is colocalized with nNOS (221, 246, 271). The role of cGKI in nociception was recently studied using the model of conventional cGKI knockout mice. These mutants showed a reduction of Formalin-evoked nociception and inflammatory hyperalgesia induced by zymosan injected into the hindpaw (271). However, their reactions to innocuous tactile and acute thermal stimuli applied to the noninflamed paws were normal, i.e., cGKI independent. In agreement with previous results (156) obtained with the mollusk *Aplysia*, these findings suggest a specific role for cGKI in the development and maintenance of hyperexcitability of nociceptive neurons, i.e., mechanisms thought to contribute to neuropathic pain in humans. It is important to note that the spinal cord of cGKI knockout mice is significantly smaller and contains fewer neurons than that of control mice (271). Thus it cannot be excluded that morphological alterations, presumably due to axon guidance defects of nociceptive neurons during embryogenesis (246), contributed to the reduced nociception observed in cGKI null mutants (271). Furthermore, reduced nociception might

be secondary to other severe phenotypes observed in conventional cGKI knockout mice.

The mechanisms involved in the transduction of cGKI activity into altered nociception are not clear. cGKI might regulate the synthesis of substance P, a major pain-promoting factor released by nociceptive nerve terminals. Indeed, the corresponding immunoreactivity was significantly reduced in the spinal cord of cGKI-deficient mice (271). Long-term sensitization of the somatosensory system through the cGMP/cGKI pathway may involve transcription-dependent memory-like mechanisms similar to those described for synaptic plasticity in the central nervous system (131, 156). In nociceptive neurons of *Aplysia*, cGKI is located in axons, where it is activated by nerve injury. In the activated state, cGKI is transported retrogradely to the cell body and phosphorylates a mitogen-activated protein kinase (MAPK). This triggers MAPK translocation into the nucleus and subsequent alterations in gene expression (267). The role of this mechanism in mammalian pain processing needs to be validated in future studies.

IV. cGMP-DEPENDENT PROTEIN KINASE SIGNALING IN THE CARDIOVASCULAR SYSTEM

Pharmacological and genetic studies have demonstrated that signaling cascades initiated by NO and natriuretic peptides (NPs) play an important role in cardiovascular health and disease (55, 85, 106, 115, 165, 179). NO and ANP relax small arteries and arterioles resulting in a decreased blood pressure. NO prevents acute vasoconstriction and thrombosis. In addition, NO/NP signaling modulates cardiac and vascular remodeling processes that are associated with the pathogenesis of chronic disorders, such as congestive heart failure and atherosclerosis. Most NO effects are protective and contribute to the maintenance of cardiovascular homeostasis, whereas others might be deleterious and promote cardiovascular pathology (64, 106, 200). The molecular mechanisms of cardiovascular NO/NP signaling are not well understood, but it is currently accepted that many effects are mediated, at least in part, via cGMP-dependent pathways (68, 77, 143, 236). cGMP-elevating drugs are successfully used in humans, for instance, the NO donor glyceryl trinitrate for the treatment of angina pectoris (208), the BNP analog nesiritide in heart failure (16), and the PDE5 inhibitor sildenafil for the treatment of erectile dysfunction and pulmonary hypertension (161). The cGKI is the major cGK expressed in the cardiovascular system including vascular smooth muscle cells (VSMCs), platelets, and cardiomyocytes (68). Conventional and tissue-specific conditional deletion of the cGKI gene has identified cGKI as an important mediator of many, but not all, cardiovascular effects of NO and NPs (Table 2). cGKI contributes not only to beneficial effects of these signaling molecules but may be also involved in potentially deleterious effects.

A. Vasorelaxation

The contractile state of VSMCs is regulated dynamically by hormonal and neural inputs. Contraction and relaxation of VSMCs is initiated by a rise and fall of the cytosolic calcium concentration ($[Ca^{2+}]_i$), respectively. $[Ca^{2+}]_i$ can be increased via two mechanisms (Fig. 2): *1*) Ca^{2+} release from intracellular stores via IP_3 or ryanodine receptors and *2*) influx of extracellular Ca^{2+} via voltage-dependent and -independent Ca^{2+} channels. The rise in $[Ca^{2+}]_i$ activates the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK), which phosphorylates the regulatory myosin light chain (MLC) leading to activation of myosin ATPase, actomyosin cross-bridging, and an increase in tension. Relaxation occurs when $[Ca^{2+}]_i$ decreases resulting in inactivation of MLCK and dephosphorylation of the MLCs by MLC phosphatase (MLCP). Smooth muscle contractility can also be modulated at constant $[Ca^{2+}]_i$. The Rho/Rho kinase pathway inhibits MLCP activity leading to increased levels of phosphorylated MLCs and tension at a given $[Ca^{2+}]_i$, i.e., Ca^{2+} sensitization of contraction (259). Thus the contractile state of the smooth muscle cell is determined by the level of MLC phosphorylation, which in turn is regulated by signaling pathways that affect the balance of MLCK and MLCP activity.

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 (134, 212, 241). cGKI inhibits both hormone receptor-triggered (212) and depolarization-induced contraction (87) by interfering with phospholipase C activation (302), by lowering $[Ca^{2+}]_i$ (65, 87, 212), by decreasing the Ca^{2+} sensitivity of contraction (21), and by unknown mechanisms. VSMCs express both cGKIα and cGKIβ (65, 86, 127, 296). 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 (244). Phosphorylation of IRAG by cGKIβ inhibits IP_3-induced Ca^{2+} release from intracellular stores in transfected COS cells and in smooth muscle cells (5, 87, 244). Recently, mice have been generated expressing a mutated IRAG protein that does not interact with the IP_3 receptor (87). In aortic smooth muscle of 9-wk-old and older IRAG mutants, cGMP suppressed neither hormone-induced increases in $[Ca^{2+}]_i$ nor smooth muscle contraction, indicating that the cGKIβ/IRAG/IP_3 receptor pathway inhibits hormone receptor-triggered intracellular Ca^{2+} release and contraction in vivo. However, this pathway is not

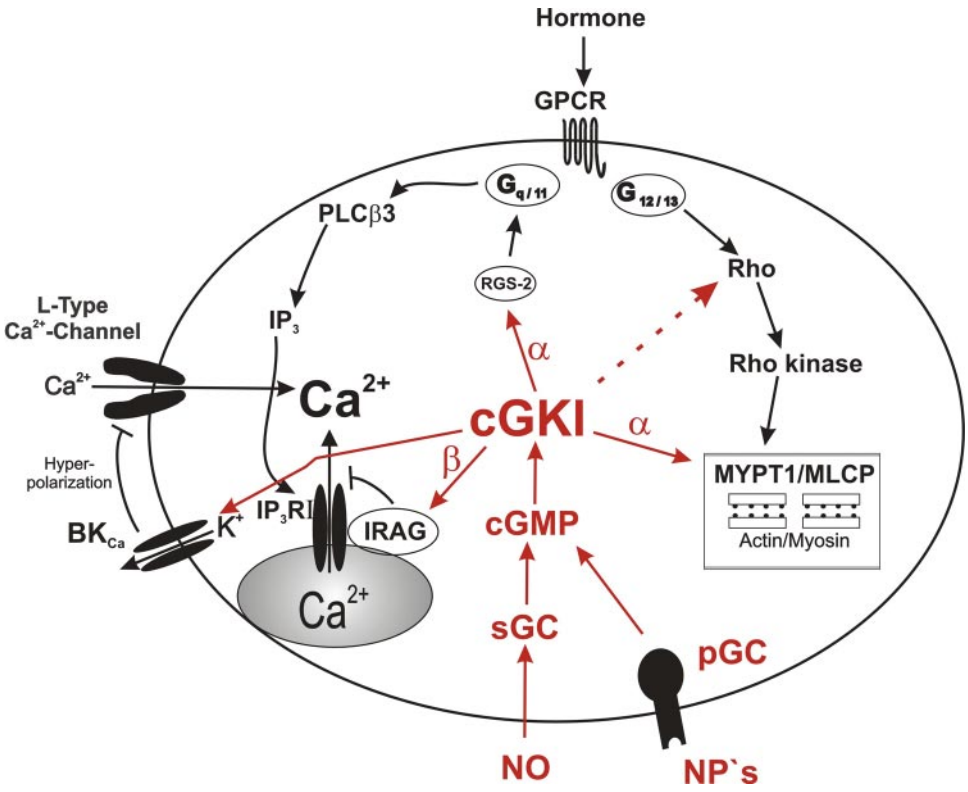

FIG. 2. cGKI-dependent relaxation mechanisms of vascular smooth muscle. Dashed lines, pathways that have not been proven in intact animal tissues; solid lines, mechanisms verified in intact animal tissue. Abbreviations are as in text.

involved in cGKI-mediated inhibition of contraction initiated by potassium depolarization and activation of voltage-dependent Ca^{2+} channels (87).

Additional important cGKI targets that contribute to vasorelaxation have been identified (Table 1). cGKI increases the open probability of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (229, 241), either by direct phosphorylation (4, 79) or indirectly via regulation of a protein phosphatase (293, 310). Opening of BK_{Ca} channels results in hyperpolarization of the membrane and closing of voltage-dependent Ca^{2+} channels, thereby reducing Ca^{2+} influx. The cGKI may also activate the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) by phosphorylation of the SERCA regulator phospholamban (136, 222, 238). Increased SERCA activity promotes Ca^{2+} reuptake into the sarcoplasmic/endoplasmic reticulum. The in vivo significance of the phosphorylation of these two proteins is unclear, because deletion of the BK_{Ca} gene (240) and the phospholamban gene (148) affected NO/cGMP-dependent vasorelaxation only marginally. cGKI may attenuate hormone receptor-activated contraction by inhibition of phospholipase C activity and IP_3 synthesis (103, 234), through phosphorylation of some receptors (287), the regulator of G protein signaling (RGS) proteins (209, 270), or phospholipase Cβ (302). Reconstitution experiments with cGKI-deficient VSMCs indicated that the inhibition of hormone receptor-stimulated Ca^{2+} release can be mediated by the cGKIα isoform (65). Recently, it has been shown that the cGKIα isoform binds, phosphorylates, and activates RGS2, which terminates signaling by G_q-coupled receptors for contractile agonists (270). 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 (266). Another target for cGKI-mediated vasorelaxation is the MLCP (151, 268). The cGKIα isoform interacts with MYPT1 and activates MLCP (268, 300). 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. Alternative targets of cGKI have been reported (20, 137, 243, 285). However, the physiology of the phosphorylation of these proteins remains to be established. The presented results suggest that cGKI inhibits receptor-induced vascular smooth muscle contraction by multiple mechanisms including the cGKIβ/IRAG, the cGKIα/RGS2, and the cGKIα/MLCP signaling pathway. The mechanism(s) by which cGKI interferes with potassium-induced contraction remains unsolved. It is likely that the individual contribution of each pathway to cGKI-mediated relaxation varies with the type, the function, and the contractile status of the vessel.

Targeted inactivation of the genes encoding eNOS (113, 252), ANP (123), or the ANP receptor GC-A (168, 201) caused systemic hypertension. In contrast, global deletion of cGKI resulted in multimorbid animals that had an elevated blood pressure at young age (~32 days) but not at older age (~43 days) (212). These results were confirmed and extended by a recent study (134). Although

NO-induced dilations of arterioles and pressure drops were impaired in cGKI knockout mice, baseline blood pressure was similar in 6- to 8-wk-old cGKI$^{-/-}$ and wild-type mice. Interestingly, acetylcholine-induced vasodilation and pressure drops were unaffected in cGKI mutants, supporting the notion that acetylcholine signals in mice through mechanisms not involving cGKI. In line with these results, the mutation of several downstream effectors of cGKI signaling, such as IRAG (87), the BK_{Ca} channel (240) and phospholamban (148) affected blood pressure only marginally. However, and in contrast to this notion, a recent report suggests that mice expressing a mutated cGKIα protein that is unable to interact with its target MYPT1 are hypertensive (183).

B. Vascular Remodeling

In addition to vasodilation, NO/cGMP signaling is involved in the development of vasculoproliferative disorders, such as restenosis and atherosclerosis. The analysis of transgenic mice showed that NO can both promote (43, 49, 141, 206, 251, 253, 276) and inhibit (37, 133, 135, 142, 191, 231) pathological vascular remodeling. These findings might explain why NO-generating drugs have not been reported to limit the progression of atherosclerosis or restenosis in humans (146). The opposing effects of NO on vascular remodeling might depend on the spatiotemporal profile of its production (cellular source, time, and quantity) and are probably mediated by different cellular and molecular mechanisms (219).

A key process in vascular remodeling is the phenotypic modulation of VSMCs from contractile to proliferating/dedifferentiated cells (15, 57, 205, 250). High concentrations of NO inhibit VSMC growth in vitro; however, the evidence for an involvement of cGMP and cGKI in the cytostatic effect of NO is inconsistent (116). Pharmacological studies have reported divergent effects of cGMP on the in vitro growth of VSMCs (140, 239). In many studies, cGMP inhibited the growth of subcultured (repeatedly passaged) VSMCs. It is unlikely that the antiproliferative effect of cGMP was mediated by cGKI, because its overexpression did not alter the proliferation of subcultured cells (45). However, cGMP and low concentrations of NO stimulated the growth of primary VSMCs (96, 297). Remarkably, the growth-promoting effect of cGMP was absent in primary VSMCs isolated from cGKI-deficient mice, whereas the cytostatic effect of NO was preserved (297) (Fig. 3). These results demonstrate that activation of cGKI promotes the growth of primary VSMCs, but is not involved in the antiproliferative effect of NO. Interestingly, cGMP slightly inhibited the growth of cGKI-deficient cells. This cGKI-independent effect of cGMP might be caused by cross-activation of the cAMP-dependent protein kinase (cAK), a known antiproliferative pathway (140). Indeed, cAMP treatment clearly suppressed the growth of both wild-type and cGKI$^{-/-}$ cells (Fig. 3). Thus the antiproliferative effects of cGMP and cGMP-elevating drugs on subcultured VSMCs might be mediated by cAK but not cGKI (64, 203). In contrast to primary cells, subcultured cells seem to be refractory to growth stimulation via the cGMP/cGKI pathway, perhaps because repeated passaging of VSMCs results in deregulation of the cGMP/cGKI pathway (26) or in more general alterations in proliferative responses.

The in vitro analysis of VSMCs led to the speculation that cGMP/cGKI signaling might also contribute to the phenotypic modulation and growth of VSMCs during pathological vascular remodeling in vivo. To test this hypothesis, the consequences of postnatal smooth muscle-specific cGKI gene inactivation were studied in hypercholesteremic ApoE-deficient mice, a mouse model of atherosclerosis (216, 308). By using a combination of Cre/lox-mediated cGKI ablation and cell marking, the fate of wild-type and cGKI-deficient VSMCs was followed during the development of atherosclerotic plaques (297). In line with the in vitro analysis of primary VSMCs, the smooth muscle-specific cGKI knockout mice showed an impaired development of VSMC-derived plaque cells and a significantly decreased lesion area. These results indicate that signaling mechanisms involving cGKI in VSMCs promote the growth of atherosclerotic plaques in vivo. The NO/cGMP pathway has also been implicated in restenosis after arterial injury, another vasculoproliferative process that involves VSMCs. Adenoviral gene transfer of the kinase domain of cGKI reduced restenosis after vascular injury in rats and pigs (254). However, this cGKI construct displays constitutive kinase activity and lacks the NH_2 terminus that specifies substrate selection (244, 268) and, therefore, possibly has effects apart from the cGMP-regulated holoenzyme. Surprisingly, gene transfer of the wild-type cGKIβ isoform had no effect on resteno-

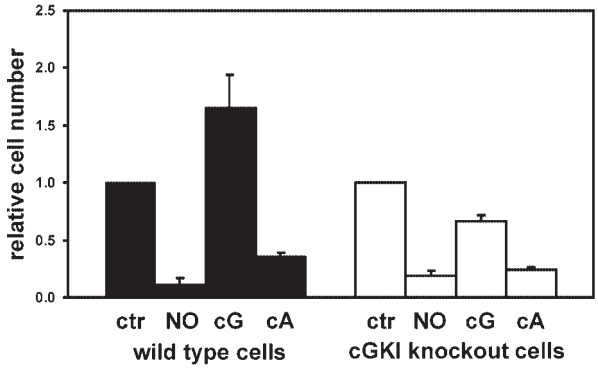

FIG. 3. Effects of NO, cGMP, and cAMP on the growth of primary vascular smooth muscle cells. Cells were isolated from aortas of wild-type (solid bars) or cGKI$^{-/-}$ (open bars) mice and grown in serum-containing medium under control conditions (ctr) and in the presence of 100 μM DETA-NO (NO), 100 μM 8-bromo-cGMP (cG), or 100 μM 8-bromo-cAMP (cA) for 96 h.

sis (254), suggesting that cGMP/cGKI signaling is not critically involved in restenosis. This conclusion is also supported by the finding that smooth muscle-specific deletion of cGKI did not affect restenosis in response to carotid ligation in normolipidemic mice (171). Thus the role of the smooth muscle cGMP/cGKI pathway in vascular remodeling might be context specific, being more important in atherosclerosis on a hyperlipidemic background than in restenosis under normolipidemic conditions.

Vascular proliferation is also involved in postnatal neovascularization, which is induced by NO through both angiogenesis (the development of new blood vessels derived from existing vessels) and vasculogenesis (blood vessel formation de novo from progenitor cells) (56). Ischemia-induced angiogenesis was potentiated in transgenic mice overexpressing cGKIα and attenuated in cGKI null mutants (304). Thus it appears that cGKI mediates, at least in part, the stimulatory effect of NO in pathological angiogenesis, a hallmark of cancer and various ischemic and inflammatory diseases (33). It will be interesting to study whether cGKI also contributes to NO-induced postnatal vasculogenesis, perhaps by promoting the mobilization and/or proliferation of stem and progenitor cells in the bone marrow (1).

Most likely, cGKI-dependent regulation of cell growth is mediated by changes in gene expression (58, 162, 215). Several lines of evidence indicate that the cGMP/cGKI system promotes vascular cell proliferation via cross-talk with the MAPK and phosphatidylinositol-3-kinase/Akt kinase pathways (13, 111, 138, 139, 297). The above discussed mechanisms, such as the modulation of $[Ca^{2+}]_i$ (128) or Rho/Rho kinase signaling (91), might contribute to cGKI effects on gene expression and cell growth as well. Other attractive targets are transcription factors, such as the nuclear factor of activated T cells (NFAT) (90), or the actin-binding protein VASP (vasodilator-stimulated phosphoprotein), an established in vivo substrate of cGKI, whose phosphorylation status has been linked to the proliferation of VSMCs (38, 311). cGKI might modulate cell growth also through effects on cell adhesion and migration (25, 257), and apoptosis (218). Further experiments with cGK-deficient mouse models will be required to decipher the exact mechanisms of cGMP/cGKI-regulated vascular growth. Collectively, the in vitro and in vivo data suggest that cGMP/cGKI signaling promotes a variety of vasculoproliferative processes under physiological and pathological conditions.

C. Platelet Function

Platelet adhesion and aggregation at the surface of vessels is central to the pathogenesis of atherogenesis and thrombosis. Prostacyclin and NO raise platelet cAMP and cGMP levels, respectively, and inhibit platelet activation. Remarkably, platelets contain high concentrations of cGKIβ and its substrates VASP and IRAG (86, 127, 166). NO/cGMP failed to inhibit aggregation and activation of the fibrinogen receptor in platelets deficient in either cGKI (178) or IRAG (7), whereas platelet aggregation and activation of the fibrinogen receptor were not affected by the deletion of VASP (98). Mouse mutants for cGKI (178), VASP (177), or IRAG (7) showed impaired NO/cGMP-dependent inhibition of platelet aggregation in vivo. These findings suggest that NO signals via cGMP and cGKI to inhibit platelet activation and that phosphorylation of both VASP and IRAG can mediate platelet inhibition in vivo. In addition, NO and cGMP might use cGKI-independent signaling pathways, such as cross-activation of the cAMP/cAK pathway and subsequent phosphorylation of VASP by cAK (122, 158). A recent report suggested that cGKI could initiate a biphasic response on platelets consisting of an initial transient stimulation of platelet aggregation and a subsequent inhibition that limits thrombus size (159). Platelet activation by cGKI might involve increased secretion of the platelet agonist ADP (160, 223), but the in vivo relevance of cGKI-stimulated platelet aggregation remains to be shown (82, 175).

D. Cardiac Contractility and Remodeling

NO and NPs modulate cardiac contractility and remodeling. The relative importance of NO effects mediated by cGMP-dependent and cGMP-independent pathways is discussed controversially (94, 179). 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 (291). However, the NO/cGMP/cGKI pathway does not appear to be involved in the negative inotropic action of acetylcholine (89, 280, 291). Cardiomyocyte-directed overexpression of cGKIα augmented NO/cGMP inhibition but not muscarinic inhibition of L-type Ca^{2+} channel activity (248). 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 (299). Thus it appears that cardiac contractility is inhibited by NO-stimulated cGMP, but increased by CNP-stimulated cGMP, and both effects are mediated via cGKI. The dual effects of cGKI on cardiac contractility might be related to distinct subcellular microdomains of NO/cGMP/cGKI versus CNP/cGMP/cGKI signaling.

The development of cardiac hypertrophy and congestive heart failure is associated with the increased expres-

sion of several fetal genes such as ANP and BNP (264). Mice lacking ANP or the ANP receptor GC-A develop pressure-independent cardiac hypertrophy (110, 123, 129, 132, 201). The hypertrophic response of cultured neonatal rat ventricular myocytes to α_1-adrenergic stimulation is suppressed by ANP, NO, or cGMP (31). These results indicate that stimulation of cGMP synthesis by ANP or NO inhibits cardiomyocyte hypertrophy. Whether or not the antihypertrophic effect of cGMP is mediated by cGKI is presently unclear. Adenoviral overexpression of cGKI inhibits myocyte hypertrophy in vitro (298), at least in part via inhibition of the calcineurin-NFAT pathway (71). However, neither global nor cardiomyocyte-specific ablation of cGKI affected the development of cardiac hypertrophy under basal conditions or in response to pressure overload (68). Recently, it was shown that administration of sildenafil suppresses the development of cardiac hypertrophy in response to pressure overload and can even reverse preestablished cardiac enlargement in the mouse (269). Surprisingly, the potent antihypertrophic 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 antihypertrophic action of sildenafil is not clear.

V. cGMP-DEPENDENT PROTEIN KINASE SIGNALING IN OTHER ORGANS

A. Urinary Bladder

The cGKI is highly expressed in the bladder of various animal species. Deletion of cGKI affected the bladder function in mice as evidenced by an altered micturition, reduced rhythmic contractility, and increased bladder volume (210). Bladder morphology and weight were unaffected by the deletion. In the urinary duct, cGKI deletion impaired NO/cGMP-dependent relaxation and led to hyperactive voiding (210). Interestingly, the relaxation of urethra induced by forskolin that stimulates cAMP synthesis was impaired in cGKI-deficient mice, indicating a cross-talk between cAMP and cGKI. Two targets of cGKI are present in bladder smooth muscle: phospholamban and BK_{Ca} channels. Whereas deletion of phospholamban had only mild effects on the control of bladder tone (197), major disturbances were reported for animals with a deleted BK_{Ca} channel gene (184). The BK_{Ca} channel may be a major target for cGKI in bladder smooth muscle.

B. Gastrointestinal Tract

An important element controlling the motor function of the gastrointestinal tract is the enteric nervous system that contains adrenergic, cholinergic, and nonadrenergic noncholinergic (NANC) neurons, which express nNOS and release NO. Deletion of nNOS impairs gastrointestinal motility and induces gastric stasis and pylorus stenosis (112, 176). cGKI is expressed ubiquitously in gastrointestinal smooth muscle including stomach, the small and large intestines, and the cecum (86). Furthermore, cGKI might be expressed in the enteric nervous system including interstitial Cajal pacemaker cells (212, 237). cGKI knockout mice exhibited a strongly disturbed gastrointestinal motility resulting in a slow passage of food (212). The gastrointestinal tract of these mice showed a dilation of the stomach, stenosis and hypertrophy of the pylorus, dilation of duodenum and cecum, and contraction of the ileocecal region (198, 212). A comparable phenotype was found in mutant $IRAG^{\Delta 12/\Delta 12}$ mice, which have a deleted exon 12 of the IRAG gene (87). The cGMP-dependent relaxation of intestinal smooth muscle strips precontracted with carbachol was abolished in $cGKI^{-/-}$ and the $IRAG^{\Delta 12/\Delta 12}$ mice.

The gastrointestinal motility of $cGKI^{-/-}$ and $IRAG^{\Delta 12/\Delta 12}$ mice is disturbed, because these molecules interfere with the Ca^{2+}-dependent and Ca^{2+}-independent regulation of contraction. Intestinal smooth muscle expresses several potential cGKI substrates, including RhoA, MYPT1, and telokin, which are part of the pathway modulating Ca^{2+} sensitization of contraction (40, 61, 259, 300). Therefore, it was not surprising that deletion of cGKI affected the activity of myosin phosphatase and contraction at constant $[Ca^{2+}]_i$ (21). In contrast, the $IRAG^{\Delta 12/\Delta 12}$ affected only the IP_3-dependent Ca^{2+} release, supporting again the notion that cGKI affects Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (87). Interestingly, cGMP-dependent relaxation of K^+/depolarization-induced contraction was abolished in $cGKI^{-/-}$ but not in $IRAG^{\Delta 12/\Delta 12}$ muscle strips (87), suggesting that cGKI can inhibit intestinal smooth muscle contraction by additional pathways that do not require signaling through IRAG.

Gastrointestinal function is also regulated by cGKII (211). cGKII is located in the secretory epithelium of the small intestine and stimulates chloride and water secretion possibly through phosphorylation of CFTR (279). 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 (34, 277). Stimulation of the cGMP signaling cascade by toxins causes diarrhea. The *Escherichia coli* heat-stable toxin (STa) and guanylin activate the guanylyl cyclase C and, thereby, increase water secretion in the small intestine. As expected, STa did not induce diarrhea in cGKII knockout mice (211).

C. Penis

Penile erection is induced by NO released from NANC neurons leading to cGMP synthesis and relaxation

of the corpus cavernosum smooth muscle (6). The cGMP signal is increased upon inhibition of the PDE5 by sildenafil, vardenafil, or tadalafil (44). Diminished expression of cGKI in diabetic rats was related to impaired relaxation of corpus cavernosum (35). cGKI-deficient male mice showed a strongly reduced reproduction, which was due to a defect in the relaxation of the corpus cavernosum (99). Spermatogenesis was not affected in these mice. Sperm from cGKI knockout mice fertilized eggs from wild-type mice (99).

D. Kidney and Adrenal Gland

cGMP might affect blood pressure not only directly by regulating vascular smooth muscle tone, but also indirectly by regulation of renin and aldosterone secretion (68, 144, 172, 196). Renin secretion is enhanced by NO through cGMP-dependent inhibition of cAMP hydrolysis mediated by PDE3 (144). In contrast, ACTH-dependent aldosterone secretion is inhibited by ANP through cGMP-dependent stimulation of cAMP hydrolysis mediated by PDE2 (172, 196). In addition, it was reported that cGMP analogs reduced renin secretion from isolated kidney or juxtaglomerular cells (101, 196, 247). Kidney expresses cGKI and cGKII. The cGKII is localized together with storage granules in juxtaglomerular cells (101). Renin secretion from juxtaglomerular cells was increased in cGKII-deficient mice (283). On the other hand, cGMP inhibits PDE3, a cAMP hydrolyzing enzyme, and thereby stimulates renin secretion (144). The two opposite effects of cGMP are both initiated by NO. These results are an excellent example showing that NO and cGMP can mediate effects opposite to that triggered by cGK.

The cGKII has also been detected in rat and murine zona glomerulosa cells of the adrenal gland (81), where the enzyme might regulate aldosterone secretion. The overexpression of cGKII in rat zona glomerulosa cells enhanced the production of aldosterone (81). Likewise, a low-sodium diet activating the aldosterone system enhanced expression of cGKII (81). In contrast, cGKII deletion in mice did not alter plasma aldosterone levels under basal conditions or after a low-sodium diet (S. Feil and F. Hofmann, unpublished results). In agreement with this finding, cGMP inhibits aldosterone secretion in bovine adrenals by stimulating the hydrolysis of cAMP (172, 196). Furthermore, cGKII deletion had no effect on resting blood pressure (211). Therefore, it is unlikely that the above-reported findings on cGKII are relevant for basal blood pressure regulation in mice and other animals.

E. Bone

The endochondral ossification of bones is stimulated by CNP (42). Overexpression of CNP rescued achondroplasia, which was induced by a defect in fibroblast growth factor receptor 3 signaling (305). The cGKII knockout mice are dwarfs that develop short bones due to a defect in endochondral ossification at the endochondral plate (211). This defect was not rescued by CNP overexpression, indicating that cGKII is an essential component of the pathway by which CNP stimulates endochondral ossification (186). Furthermore, cGKII-deficient rats exhibited an expanded growth plate, impaired bone healing, and an accumulation of postmitotic but nonhypertrophic cells (39). Transfection experiments showed that cGKII inhibited nuclear entry of the transcription factor Sox9, which is associated with normal chondrocyte differentiation. The results obtained in mice and rats revealed that cGKII works as a molecular switch that regulates differentiation of chondrocytes (39).

VI. FUNCTION OF cGMP-DEPENDENT PROTEIN KINASES IN THE FLY, BEE, AND WORM

cGKs were also identified in a large number of invertebrates. *Drosophila melanogaster* has two cGK genes, *dg1* and *dg2* (124). Dg2 encodes a protein kinase that is related to the mammalian cGKI gene, whereas the Dg1 protein is more homologous to cGKII (73, 124). Dg2 is localized at the plasma membrane, whereas Dg1 is found predominantly in the cytosol (173). Overexpression of both proteins in renal tubules suggested that Dg1 and Dg2 increase tubular fluid transport, a function related to cGKII activity in mammals. Furthermore, Dg2 affects development and insect behavior. Dg2 increased survival during hypoxia and induced tracheal branching, which is an analogous process to tumor angiogenesis in mammals (295). Analysis of naturally occurring *Drosophila* variants in food-searching behavior indicated that a high activity of the dg2 kinase is a major determinant of rover versus sitter behavior and, therefore, associated with increased locomotion (202, 258). A similar situation has been identified in honey bees. A cGKI-like kinase activity is upregulated when the young bees change from hive work to foraging (14).

The invertebrate *Caenorhabditis elegans* has two genes related to the mammalian cGKs. The sequence of Egl-4 is 48.3 and 41.6% identical with cGKIα and cGKII, respectively (104). The second gene (*C0964.2*) has 41.8 and 35% identity with cGKIα and cGKII, respectively (104). Two isoforms of Egl-4, a and b, exist that are localized differentially. The a-isoform is expressed in neurons, hypodermis, and the intestine, whereas the b-isoform is found in the body wall muscles, suggesting different signaling pathways of these isoforms. Genetic interaction studies showed that cGMP/Egl-4 is a negative growth regulator that suppresses body size through DBL-1/transforming growth factor-β signaling (104, 192). Ani-

mals with an inactivating mutation in egl-4 showed an ~50% increased body volume that was caused by an elevated fluid content, whereas cell proliferation was unchanged (192). Only the a-isoform rescued the body size phenotype of egl-4 mutants, suggesting that both isoforms exhibit different functions (104).

The cGK activity of *egl-4* is also involved in the food-seeking behavior of *C. elegans*. In these animals, long distance roaming for food is associated with a decreased cGK activity (78). Furthermore, mutants of the *C. elegans* cGK gene *egl-4* exhibit olfactory defects with reduced preadaptation to odor (152). The *egl-4* gene involved in the food-seeking behavior is more closely related to mammalian cGKI than to cGKII (see Refs. 104, 193 and above), pointing to the possibility that increased cGKI activity may be related to a sedentary life-style in vertebrates.

The above studies unequivocally demonstrate that cGKs are not only involved in cardiovascular physiology and development, but regulate also complex central nervous processes. Subtle changes in cGK activities can lead to naturally occurring behavioral variants that may have significant effects on mammalian behavior.

VII. CONCLUSIONS AND FUTURE DIRECTIONS

Genetic manipulation of the cGK genes has established these protein kinases as essential molecular regulators of cardiovascular, intestinal, and neuronal functions. Details of their signaling pathways are emerging, and increasing evidence is mounting that cGKs can signal via several targets in the same cell, probably regulating different functions of a cell. The analysis of the cGK-deficient mouse models showed that NO can signal independent of cGKs. Validated examples have been established that even cGMP might use signaling pathways independent of cGKs. Thus it will be necessary to study in the future the details of various cGMP and cGK signaling pathways keeping in mind that these molecules may signal independent of each other. It will be essential to establish the role of cGKs in cell growth and in various brain functions. The results obtained with flies, bees and worms suggest that mammalian behavior may be modulated by the activity of the cGKs. cGKs are widely distributed in brain nuclei and might be essential regulators of neuronal networks. Together, the results obtained with several mouse models suggest that the cGKs are interesting drug targets (245) that might allow treatment of diseases such as excessive food intake, atherosclerosis, and pathological vessel growth.

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