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MOLECULAR PROPERTIES AND BIOLOGICAL FUNCTIONS OF cGMP-DEPENDENT PROTEIN KINASE II

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1. ABSTRACT

Type II cGMP-dependent protein kinase (cGK II) is the protein product of one of two genes coding for cGKs in mammalian genomes. cGK II has a domain structure similar to cGK I (alpha or beta) consisting of an N-terminal regulatory domain, which contains a dimerization and an autoinhibitory region, two cGMP-binding domains and a C-terminal catalytic domain. However, the position of the high and low affinity cGMP-binding-domains in cGK II are reversed in comparison to cGK I. Moreover, the isoenzymes exhibit a different affinity towards various membrane permeable cGMP-analogs, allowing differentiation between the cGKs. Type II cGK is bound to the membrane by a myristoyl moiety. It has a distinct function and an expression pattern distinct from that of cGKI, being expressed predominantly in intestine, brain, and kidney. It is involved in regulating electrolyte and water secretion by epithelial tissues in response to the luminocrinic hormones guanylin and uroguanylin and in the secretory diarrhea provoked by heat-stable enterotoxins. Type II cGK also plays a role in the regulation of endochondral ossification by C-type natriuretic peptide, in renin secretion by the kidney, aldosterone secretion by the adrenal, and in the adjustment of the biological clock.

2. INTRODUCTION

The existence of a second isoform of cGMP-dependent protein kinase (cGK) was anticipated in the early 1980's by the detection of a membrane-bound cGMP receptor in intestinal epithelium (1). After solubilization and purification, the intestinal cGMP receptor turned out to be a protein kinase, possessing a number of characteristics clearly distinguishing it from the cytosolic cGK type I isolated from most other tissues. This novel particulate cGK together with an intestine-specific guanylyl cyclase identified in the same time period, constituted a unique cGMP pathway, activated by an heat-stable enterotoxin from pathogenic strains of *Escherichia* coli or by guanylin and uroguanylin, intestinal hormones involved in fluid homeostasis (2). A gene encoding a novel cGMP-dependent protein kinase, different from cGK I was cloned subsequently from brain tissue and intestine (3,4), and the protein product was shown to be identical to the intestinal cGK (4), demonstrating a more general expression of this enzyme than previously anticipated. Analysis of the phenotype of knock-out mice confirmed that cGK II has other functions *in vivo* aside regulation of intestinal fluid transport, that are clearly different from the functions of the type I isoform (5).

3. THE GENE FOR cGK II.

The gene encoding cGK II has been analyzed from the human (PRKG2) (6), mouse and rat genome (Prkg2; genome pojects). The gene is at least 125 kb long and consists of 19 exons, which are very similar between the three species, although the intron lengths vary considerably. In humans PRKG2 is located on chromosome 4, whereas the Prkg2 gene is located on chromosome 5 and 14 in mouse and rat, respectively. The gene structure of PRKG2 corresponds well to that of the type I cGK. On the basis of the gene structure Witczak et al. concluded that the two mammalian cGKs are more related to each other than to the Drosophila melanogaster DG2 and DG1 genes encoding fly cGKs (6). The translation start codon of cGK II is located on exon 2, meaning that all species have at least one non-translated exon. The dimensions of this first exon are unknown as the transcription initiation site is poorly characterized (6). Likewise no experimental analysis of the 5' upstream gene regulatory sequences has been reported. To date only one splice variant has been described. In this variant with unknown function, an alternative 5' splice site in exon 11 is used, generating an inactive protein, which lacks 29 amino acids of the catalytic domain and inhibits full length cGKII (7).

4. EXPRESSION OF cGK II

Based on protein or mRNA detection in whole tissues from rat and mouse, cGK II was found to be expressed most abundantly in the intestine (4,5). From the other tissues examined, brain, kidney and prostate also stained positive for cGK II (4,5,8), whereas mouse additionally shows a clear cGK II signal in lungs (3,5). Furthermore, cGK II was detected in chondrocytes in the .growth plate of bones (5), in zona glomerulosa cells in the adrenal cortex (9), in Clara cells in the lung (10) and in epithelial cells of the gallbladder (11), pancreas (12), and salivary gland (13). Finally, mRNA expression of cGK II was observed in fibroblasts (14), stromal cells of the prostate (15) and various cell types of the mouse eye (16).

In the intestine, cGK II expression is limited to the epithelial cells lining the gut lumen (17). Along the gastro-intestinal tract, cGK II mRNA was found in the small intestine (duodenum, jejunum and ileum), the cecum and proximal colon but not in the stomach or the distal colon. At the protein level, cGK II was enriched in the proximal part of the small intestine. In the jejunum, cGK II protein was most abundant in cells from the tip of the villus, and its expression decreased along the crypt-villus axis in the direction of the crypts. It was estimated that rat jejunal villus cells contain 0.2-0.4 microgram of cGK II per mg of protein (18). This was mainly concentrated in the brush border area of the cell, although the basolatereral membrane was also found to contain cGK II (Vaandrager, unpublished data). The presence of cGK II in the intestinal tract was confirmed in humans (17), rabbits and pigs (1). The expression of cGK II in intestinal cell lines may vary as both the presence and absence of a cGK II signal was reported for the T84 human colon carcinoma cells (17,19), and expression of cGK II is often lost upon passaging (20).

In bone the level of cGK II was found to vary with the differentiation state of the chondrocytes, as cGK II was predominantly localized in the growth plate in the border between the proliferating and hypertrophic chondrocytes (5). In the latter cell type cGK I was the main isoform. Expression of cGK II in brain is much more widespread compared to the type I isoform, which is expressed most abundantly in the cerebellum. Regions in the brain containing high levels of cGK II mRNA include the thalamus, cerebral cortex, septum, amygdale, olfactory bulb, and various brainstem nuclei (21). Immunocytochemistry revealed that cGK II protein was enriched in the dendritic or axonal outgrowth of neuronal cells rather than in the cell body (22). Expression of cGK II in the rat kidney was noted especially in the juxtaglomerular cells, the ascending thin limb of Henle's loop, and to a lesser extent in proximal tubules (23). In a semi-quantitative mRNA analysis in microdissected nephron sections, cGK II mRNA was found to be expressed in similar areas as the enterotoxin receptor GC-C (24). In the rabbit, cGK II was also observed in cells isolated from connecting tubules and cortical collecting ducts (20). Interestingly the levels of cGK II in the rat juxtaglomerular cells and in the ascending thin limb were upregulated in response to angiotensin receptor blockade and dehydration, respectively, whereas cGK II levels in adrenal zona glomerulosa cells were up regulated by a low salt diet (9,23).

In epithelial tissues the heat-stable enterotoxin receptor/guanylyl cyclase C was commonly found to be co-expressed with cGK II. However, in other tissues, in particular in brain and bone, the cGMP required to activate cGK II is likely to be generated by the NO-sensitive soluble guanylyl cyclase GC-S and/or the natriuretic peptide receptor-coupled guanylyl cyclases GC-A or GC-B (22,23,25,26).

5. STRUCTURE OF cGK II

5.1. Domain structure

Type II cGK contains 762 amino acids with a molecular mass of approximately 87 kDa. As shown in (Figure 1A), cGK II is composed of three functionally distinct domains (27):

- A N-terminal domain comprising a leucine zipper motif involved in dimerization and an autoinhibitory/pseudosubstrate region 'RRGAKAGVSAEP' (amino acids 118-130) that interacts with the catalytic domain in the absence of cGMP. Except for these two motifs, the N-terminal domain of cGK II has a low homology to the N-terminus of the cGK Ialpha or Ibeta isozyme (27). In contast to cGK I, type II has a myristoyl moiety attached to its N-terminus, which was shown to play a role in the membrane localization of cGK II (28).

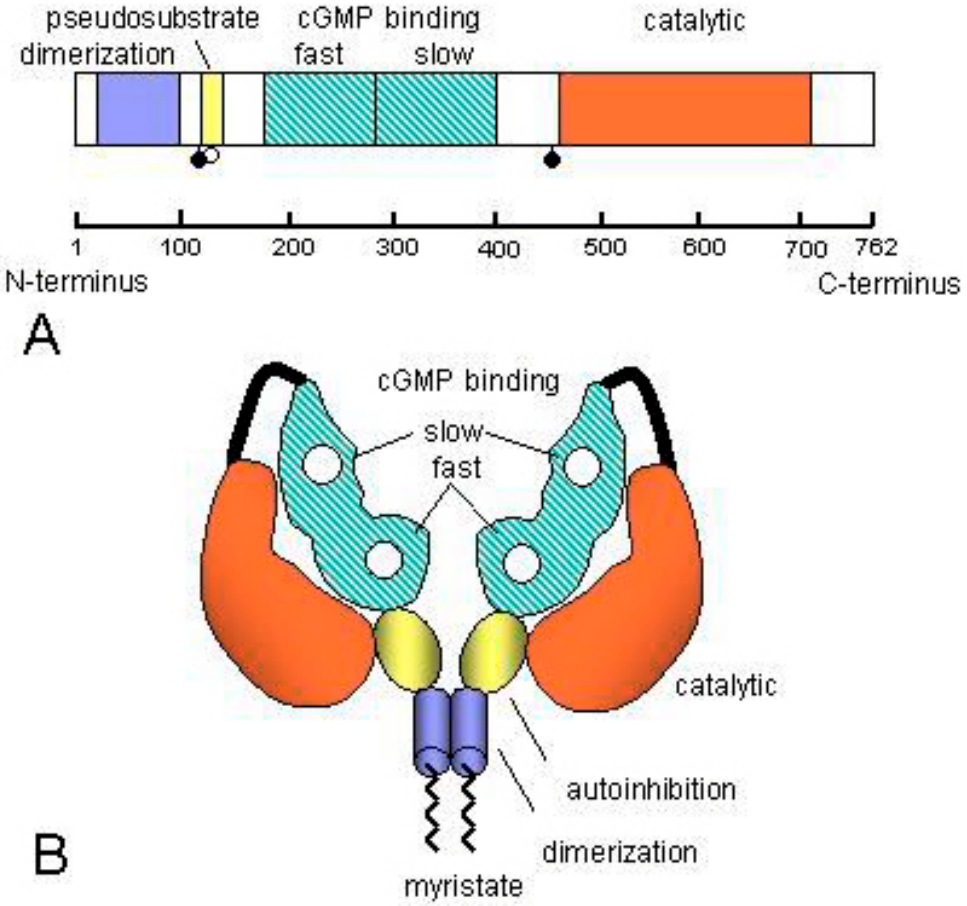

Figure 1. Model of cGK II. A, Subdivision of cGK II in various functional domains as described in chapter 5.1. Ruler indicates the position of the amino acids. Closed circles represent major autophosphorylation sites and the open circle indicates the minor, but functionally relevant, autophosphorylated residue Ser^{126}. B, Schematic model of the cGK II dimer in the inactive state i.e. in the absence of cGMP.

- A regulatory domain consisting of two cyclic nucleotide binding sites in tandem. This domain is almost 50% identical to its counterpart in cGK I and also shows a large homology to the regulatory subunit of cAK I or II (27). A Thr at position 243 in the N-terminal cGMP binding site and a Ser at position 366 in the C-terminal site are thought to confer specificity for cGMP over cAMP as these residues may interact with the C2 amino group of the guanine ring (29). Type I cGK has threonines in both the analogous positions where cAMP-dependent protein kinase has Ala residues. As discussed below (section 6.1.2), the relative affinities of the N-terminal and C-terminal binding sites for cGMP are reversed in cGK II as compared to cGK I (29). In cGK II, the N-terminal cGMP binding site is the fast (i.e rapidly dissociating)/ low affinity site, whereas the N-terminal site in cGK I is the slow/high affinity site.

- A catalytic domain containing the ATP and the substrate binding sites. This domain has the highest homology to the catalytic domain of cGK I (almost 70 % identity), and is also clearly related to the analogous domain of other serine/threonine kinases such as cAK (27). As observed for many other protein kinases, mutation of a conserved lysine in the ATP binding site at position 482 of cGK II (K482A) rendered cGK II catalytically inactive (9).

- In addition, cGK II has a functionally ill defined sequence of approximately 50 amino acids in between the regulatory and the catalytic domain, which is lacking in cGK I, and a C-terminal domain of unknown function, which is conserved between cGK I and II.

5.2. Subunit structure

As shown in (Figure 1B), cGK II is a homodimeric protein (30,31). The two monomers are linked by the N-terminal leucine zipper structure, as proteolytic removal of the first 100 amino acids yields a monomeric protein (31). It is generally assumed that the monomers are arranged in parallel. In this way, it is easy to envisage that both the myristic acids attached to the N-terminus can insert in the membrane. The precise architecture of cGK II is as yet unknown. In analogy with the proposed structure for the cAK holoenzyme (32) or

Table 1. cGK II substrates

Substrate	Sequence	Km (μM)	Selectivity cGK II/cGK I
Synthetic peptides			
• CREBtide	KRREILSRRPSYR	16	2.3
• IP_3Rtide	GRRESLTSFG	10	1.1
• VASPtide	RRKVSKQE	100	0.65
• BPDEtide	RKISASEFDRPLR	100	0.47
• Kemptide	LRRASLG	70	2.0 – 0.4
• H2Btide	RKRSRAE	305	0.05
Proteins			
• StAR $Ser^{55/56}$	QVRRRSSLLGSQ		
• StAR Ser^{99}	GWKKESQQENG		
• PTPS Ser^{19}	VSRRISFSASHRL		
Autophosphorylation of cGK II			
• Ser^{110}, Ser^{114}, Ser^{117}/Thr^{109}	RKTSGLVSLHS		
• Ser^{126}	RRGAKAGVSAEP		
• Ser^{445}	VARFSSTSPF		

Sequence of various synthetic peptides or protein residues phosphorylated by cGK II *in vitro*. Phosphorylated residues are underlined. StAR, Steroidogenic acute regulatory protein; PTPS, 6-pyruvoyltetrahydropterin synthase. Data on synthetic peptides are derived from refs 30 and 39, and on protein substrates and autophosphorylation from refs 9, 37 and 49.

cGK I (33), the N-terminus and the regulatory domain probably form an elongated structure on which the catalytic domain folds back. In the absence of cyclic nucleotides, the catalytic head interacts with the pseudosubstrate region in the N-terminal domain and probably with sequences in the regulatory domain (34) (Figure 1B).

5.3. Post translational modifications

5.3.1. Myristoylation

After biosynthesis, cGK II is myristoylated on the penultimate glycine by a N-myristoyl transferase (28). Mutation of Gly^2 to Ala or pharmacological inhibition of N-myristoylation, caused a translocation of cGK II from the plasma membrane to the cytosol (28). The absence of the myristoyl group did not affect the specific enzyme activity or the K_a for cGMP and only slightly enhanced the thermostability of cGK II. However, nonmyristoylated cGK II demonstrated a severely impaired function, as it was unable to activate physiologically important cGK II substrates, such as the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel or the renal epithelial Ca^+ channel (TRPV5/ECAC1) in the plasma membrane of intact cells (20,35).

5.3.2. (Auto)phosphorylation

cGK II was originally discovered as a major phosphoprotein in intestinal brush border membranes upon addition of cGMP and radiolabelled ATP (36). In the presence of (sub)millimolar concentration of ATP, cyclic nucleotides were shown to stimulate the autophosphorylation of type II (cGK) on multiple sites to stoichiometries of 4-6 phosphates per cGK II monomer both *in vitro* after purification and in intact cells (34,37). cGMP rapidly stimulated the phosphorylation of residues Ser^{110} and Ser^{114}, and, at a lower rate, of Ser^{126} and Thr^{109} or Ser^{117}, all located in the auto-inhibitory region (Figure 1A; Table 1). In addition Ser^{445} was found to be phosphorylated in a cGMP-dependent manner (37). At low ATP concentrations (μmolar), cAMP was even more potent than cGMP in stimulating cGK II autophosphorylation (34,37) and induced phosphorylation of sites not phosphorylated in the presence of cGMP.

The relatively slow cGMP-stimulated autophosphorylation of Ser^{126} renders cGK II constitutively active, and therefore may serve a memory function, whereas the fast autophosphorylation of the serines 110, 114 and 445 has no effect on the activity of cGK II. The regulation of the activity of cGK II by autophosphorylation is discussed in more detail in chapter 6.3. Apart from the cyclic nucleotide-stimulated phosphorylation, cGK II is also phosphorylated in the basal state on several residues including Ser^{97}, Ser^{110} and most likely, as deduced from sequence comparison with cAKs and cGK I, on Thr^{609} in the catalytic domain (37). Phosphorylation of the corresponding threonine in the catalytic core of the other cyclic nucleotide-stimulated protein kinases is thought to be crucial for enzymatic activity (38).

6. REGULATION OF cGK II ACTIVITY

6.1. Regulation by cyclic nucleotides

6.1.1. Cyclic nucleotide specificity

The physiologically most relevant activator of cGK II is cGMP. Saturating concentrations of cGMP can activate cGK II *in vitro* approximately 10 fold to a maximal velocity of 1-3 μmol/min/mg protein, depending on the substrate and the concentration of ATP used (1,30,31,39). However, the concentrations of cGMP required to half-maximally stimulate cGK II *in vitro* varied considerably (from 0.04 μm to 0.8 μM) between different studies making it difficult to compare them with K_a values for cGK I alpha (0.1-0.3 μM) (30,31,39). Part of these differences might be explained by variations in purification procedures and/or assay conditions. Solubilization of recombinant rat cGK II resulted in a slight (1.6 fold) increase in its apparent K_a for cGMP. Furthermore, the concentration of ATP present in the assay was found to affect the apparent K_a of

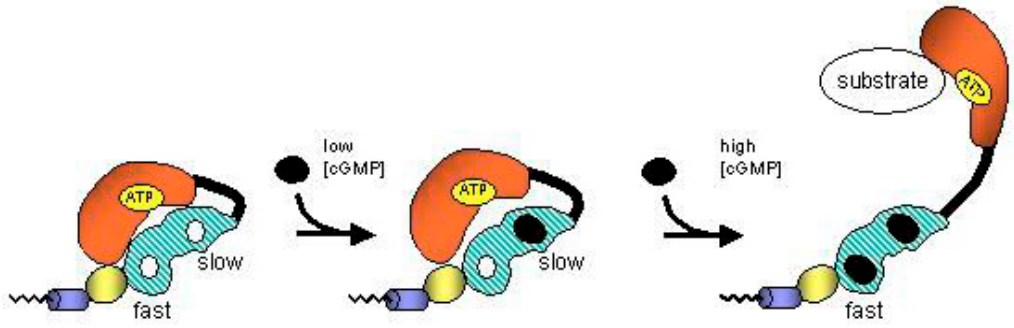

Figure 2. Tentative model illustrating the mode of activation of cGK II by cGMP (cf. ref. 29). For clarity only one monomer of the cGK II dimer is depicted, cf. (Figure 1B).

cGK II for cGMP by as much as 10-fold (31). After correction for the different ATP concentrations, the reported apparent K_a for cGMP of endogenous rat cGK II (0.36 μM at 0.3 mM ATP) was approximately 3-fold higher than that reported for recombinant rat cGK II purified from Sf9 cells (39), however 3-fold lower than that reported for either recombinant rat cGK II solubilized from COS-1 cells (28) or histidine-tagged recombinant mouse cGK II purified from Sf9 cells (30), and similar to the apparent K_a for recombinant mouse cGK II expressed in COS cells (3). Under physiological conditions in intact cells the concentration of GMP needed to evoke a full cGMP response may also depend on the local concentration of cGK II itself, which can be as high as 20 μM in the brush border compartment of intestinal epithelial cells (40).

The other naturally occurring cyclic nucleotide, cAMP, can also activate cGK II, albeit at a more than 100-fold higher concentration than cGMP (30,37,39). Since cAMP concentrations may be elevated well into the micromolar range upon hormonal stimulation, the cross-activation of cGK II by cAMP might be physiologically relevant (41). However, unequivocal proof for the occurrence of such a promiscuous regulation in native tissues has not been provided yet.

In order to demonstrate the involvement of cGK II in specific biological responses and to discriminate between cGK II and cGK I alpha or beta, several membrane permeable cyclic nucleotide analogs were developed. The effects of cGMP-analogs on endogenous rat cGK II and on recombinant cGK II purified from sf9 cells corresponded reasonably well in *in vitro* assays. In general, 8-pCPT-cGMP proved to be a more potent activator of cGK II compared to PET-cGMP or 8-Br-PET-cGMP. Interestingly, activation of cGK II by 8-pCPT-cGMP, similar to cGMP, but unlike the activation by PET-cGMP analogs, showed cooperative kinetics. In contrast, PET containing cGMP analogs are better activators of cGK I (alpha or beta) than pCPT-cGMP (30,31,39). A similar specificity of the cGK isoforms for these analogs was observed in intact cell systems, even when either recombinant cGK II or cGK Ibeta were targeted to the same substrate, i.e. the CFTR Cl^- channel (31,35). This implies that the cGMP analog specificity of cGK II, which is clearly distinct from that of of cGK I, can fortuitously be exploited to discriminate between the functional effects of each isotype both in *in vitro* assays and in intact cells. A potency order 8-pCPT-cGMP >> 8-Br-PET-cGMP > PET-cGMP would implicate a role for GK II, whereas the order 8-Br-PET-cGMP > PET-cGMP > 8-pCPT-cGMP would indicate the involvement of cGK I.

For inhibitory Rp-cGMPS analogs, a similar preference of cGK II for CPT- over PET analogs was observed. In comparison with Rp-8-Br-PET-cGMPS, Rp-8-pCPT-cGMPS showed a lower K_i value, although both compounds were able to inhibit cGMP-induced Cl^- secretion in intact intestinal epithelium (31). Except the Rp-cGMPS-analogs, no highly specific pharmacological inhibitors of cGK II are described yet, although the isoquinolene-derivative H8 and the general kinase inhibitor staurosporine were useful within a very narrow concentration range to discriminate between cGK II and cAK involvement in the activation of Cl^- secretion in intestinal eptithelium (42). The staurosporine-related compound KT5823, used with ambiguous results as an inhibitor of cGK (43), failed to inhibit the cGK II-mediated Cl^- secretion in the intestine (unpublished observations).

6.1.2. Mechanism of activation by cyclic GMP

The mechanism of cGK II activation is not fully explored yet but is thought to resemble the activation mechanism of cGK I and cAK. In the absence of cyclic nucleotides, the catalytic domain interacts with auto-inhibitory regulatory sequences, in particular with amino acids located in the so-called pseudosubstrate region, and thus is unable to interact with substrate proteins. Upon binding of cyclic nucleotides, the inhibition of the catalytic head by the regulatory regions is relieved and the catalytic domain is then free to phosphorylate substrate proteins. In the cAKs the catalytic and the regulatory domains are located on separate subunits and the catalytic domain dissociates from the regulatory subunit upon cAMP binding. In contrast, in the cGKs the catalytic domain resides on the same polypeptide chain as the regulatory domain and is thought to bend away from it, as evidenced by a substantial elongation of monomeric cGK I after binding of cGMP (33) (Figure 2).

Evidence that the pseudosubstrate domain in cGK II is required to keep the catalytic domain inactive in the absence of cGMP came from mutational studies, which showed many critical amino acids in this domain. Replacing these residues by other amino acids, rendered cGK II more sensitive to cGMP or even constitutively active (34,37) (Figure 2). The pseudosubstrate domain by itself is not sufficient to inactivate the kinase domain, as a

16-mer peptide encompassing the pseudosubstrate region (Ser^{117} to Arg ^{132}) was a poor inhibitor of cGK II. Presumably, additional interactions with residues outside the pseudosubstrate region stabilize the binding of the catalytic domain with the regulatory domain in a cGMP-dependent manner, as a full length regulatory domain of cGK II, consisting of the complete N-terminal part up to the catalytic domain (including the pseudosubstrate region and the two cGMP binding sites) was able to inhibit cGK II at nanomolar concentrations only in the absence or at sub-saturating levels of cGMP (34).

Similar to cGK I and cAK, cGK II has two homologous cyclic nucleotide binding sites showing different kinetic and functional properties (29). The sites are named 'slow' and 'fast', referring to the velocity by which they can release cGMP. Since the affinity of cGMP is inversely correlated to the 'off' rate (as the sites are thought to have similar 'on' rates), the fast site is also called the low affinity site, whereas the slow site is the high affinity cGMP-binding site. By mutating residues critical for cGMP binding in both the N-terminal site (Thr^{242} to Ala or Gly^{231} to Glu) and the C-terminal cGMP binding site (Ser^{366} to Ala or Gly^{355} to Glu) it could be demonstrated that in cGK II the N-terminal site is the fast/low affinity site, whereas the more C-terminal site is the slow/high affinity site. Interestingly, the order between these sites in cGK II is identical to the order in cAK but reversed in comparison to cGK I, in which the slow/high affinity site is the most N-terminal (29).

The two cGMP binding sites contribute to activation of cGK I and cGK II in a different manner. For cGK I occupation of the slow/high affinity (N-terminal) site alone was shown to result in a partially active kinase, which could be further activated by occupation of the fast/low affinity site at higher cGMP concentrations. In contrast it was suggested that in cGK II occupation of the slow/high affinity site alone has no effect on the capacity of cGK II to phosphorylate heterologous substrates and therefore cGMP binding to the N-terminal fast/low affinity site is required for full activation of the kinase (29)(Figure 2). However, in the presence of low concentrations of ATP, occupation of the slow/high affinity site alone is considered to be sufficient to stimulate the autophosphorylation of cGK II (31). The observation that cAMP, a poor activating ligand for the fast/low affinity site (29), stimulates cGK II autophosphorylation relatively well at low concentrations of ATP corroborates the role of the slow/high affinity site in this process.

6.2. Regulation by ATP

Raising the concentration of ATP from 10 μM to 1 mM was found to increase the concentration of cyclic nucleotides required for activation of cGK II by as much as 10-fold. A similar modulatory effect of concentrations of ATP around the K_m was observed for cGK I and cAK type I (31,44-46). This suggests a similar mode of interaction between the ATP-bound catalytic domain and the regulatory domain in all three cyclic nucleotide-dependent protein kinases, resulting in an apparent competition between ATP and cyclic nucleotides (46). However, the effect of ATP on cGMP affinity in kinase activity measurements was particularly prominent in the case of cGK II which displayed an unusually high K_m for ATP (400 μM) (31) in comparison to cAK (5 μM) (46) and cGK I (20-70 μM) (44,45). ATP also affects cGK II autophosphorylation, since at low ATP concentrations (μmolar), but not at millimolar ATP, cAMP stimulation of autophosphorylation exceeded the stimulation by cGMP, and cAMP induced phosphorylation of residues that remained unphosphorylated in the presence of cGMP (31).

6.3. Regulation by autophosphorylation

Prolonged autophosphorylation (up to several hours) of purified recombinant cGK II *in vitro* resulted in a 40-50 % increase in basal kinase activity, but its maximal cGMP-stimulated activity and the EC_{50} for cGMP remained unaltered (37). The fast phase of cyclic nucleotide-stimulated autophosphorylation of cGK II, involving serines 110, 114 and 445, has a negligible effect on its activity. However, the secondary phase, presumably involving Ser126 phosphorylation, may generate a constitutively active form of the enzyme. Ser126 is located close to the autoinhibitory domain of cGK II and therefore its phosphorylation may disturb the inhibitory interaction of this regulatory sequence with the catalytic domain (Figure 1). Slow autophosphorylation of Ser79 in cGK Iβ, situated at a position equivalent to Ser126 in cGK II, rendered the enzyme constitutively active and produced an apparent conformational change indistinguishable from the change induced by binding of cGMP to the regulatory domain (37,47). The functional significance of cGMP-stimulated autophosphorylation of cGK II is not fully elucidated yet. Evidence that it may have a memory function in vivo was provided by studies of cGK II-dependent chloride secretion in intact intestinal epithelium, showing that the Cl- secretion became partly independent of cGMP stimulation after a prolonged (3hr) exposure of the tissue to a membrane permeable cGMP analogue (37).

7. FUNCTIONS OF cGK II

7.1. cGK II substrates

As expected from a cyclic nucleotide-dependent protein kinase, cGK II regulates the activity of processes in cells in which it is expressed in a cGMP-dependent manner by phosphorylating specific target proteins on serine or threonine residues. The substrate specificity of cGK II was found to be very similar to that of both cGK I and cAK. Consequently, most cGK II substrates are also targets for the other cyclic nucleotide dependent protein kinases in vitro or in vivo, when present in the same subcellular compartment. However, as shown in (Table 1), some differences between the substrate specificity of the cGMP depependent kinases were noted in in vitro assays using specific peptide substrates. The consensus sequence for phosphorylation by cGK II derived from these studies is RRXS/T, in which R denotes basic residues, i.e. arginine or lysine, and X denotes any residue (27,30,39).

So far, a limited number of physiologically relevant cGK II substrates have been identified . The best documented cGK II substrate is the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), a highly regulated Cl^- channel involved in salt and fluid secretion

across epithelia (see section 7.3). CFTR could be phosphorylated by cGK II both in intact cells and in vitro (18,48). In the latter assays, cGK II was shown to phosphorylate CFTR on at least five sites shared by cAK and cGK I, located all in the regulatory (R) domain, and presumably including Ser700 (48). As yet the phosphorylated residues were identified only for two additional cGK II substrates: (i) the steroidogenic acute regulatory protein (StAR), involved in aldosterone synthesis in zona glomerulosa cells (see section 7.5), which was phosphorylated by cGK II in vitro at serine 55/56 and at serine 99 (Table 1) (9). Interestingly, Ser55 but not Ser99 was phosphorylated also by cAK, whereas the other cAK phosphorylation sites detected, Ser 195 (in the sequence KRRGSTCVLA) and Ser267 (in the sequence LKRKLESSPA) were not phosphorylated by cGK II; (ii) the human 6-pyruvoyltetrahydropterin synthase (PTPS) which was found to be phosphorylated in vitro by cGK II on Ser19 (49). PTSP participates in the synthesis of tetrahydropterin (BH4), an essential cofactor for the nitric oxide synthases and the aromatic amino acid hydroxylases. The physiological relevance of the cGK II mediated phosphorylation is unknown, as cGK II-/- mice had no aberrant levels of PTPS activity or tetrahydropterin synthesis. The cAK/cGK I substrates DARPP-32, G-substrate and phosphatase inibitor-1, all implicated in inhibition of protein phosphatases in nervous tissue, could also be phosphorylated in vitro by cGK II (2). Finally a number of unidentified cGK II substrate proteins were detected by in vitro phosphorylation of brain (25) and intestinal tissues, including a 25 kDa protein in the intestinal brush border membrane (50). Several other candidate substrates, including the epithelial Ca2+ channel TRPV5 (20) and the Na+/H+ exchanger NHE3 (51) do contain consensus sequences for cGK II, are colocalized with cGK II in the apical membrane, and are functionally affected by cGMP/cGK II; however, definite proof for their direct phosphorylation by cGK II is presently lacking.

7.2. cGK II anchoring proteins

In order to phosphorylate a specific protein under physiological conditions, cGK II must be present in the same subcellular location as the substrate. Removal of the N-terminal myristic acid moieties of cGK II prevented membrane binding and precluded its capacity to phosphorylate membrane-bound CFTR and its capacity to stimulate a Ca^{2+} transporter (20,35). On the other hand non-myristoylated, but not wild type cGK II, could enter the nucleus and activate the c-fos promoter in fibroblasts in response to cGMP (52). Aside targeting by lipid anchoring, many protein kinases are positioned close to their substrates by specific anchoring/scaffolding proteins. cGK II is known to be linked to the cytoskeleton by an as yet unknown protein (1). A candidate could be the PDZ domain containing anchoring protein NHERF 2/E3KARP, implicated in the regulation of the Na^+/H^+ exchanger NHE3 by cGK II (51) (see section 7.3). Sofar only one other cGK II anchoring protein has been described, i.e. the myosin heavy chain (53). The physiological function of the myosin-cGK II binding however is as yet unknown (53).

7.3. Role of cGK II as an ion transport regulator in intestinal, pancreatic and airway epithelial cells

The relative abundance of cGK II along the gastrointestinal tract in both crypt, villus and surface cells and the notion that its activator cGMP is the second messenger mediating heat-stable enterotoxin provoked secretory diarrhea, suggested a key function of cGK II in the regulation of salt and water transport in the intestine (17). On the basis of pharmacological studies using cGK II activators and inhibitors as well as functional studies in $cGK^{-/-}$ mice, a role of cGK II in the regulation of the fluidity of the intestinal contents was definitely established and was shown to involve a dual action: stimulation of Cl^- secretion through activation of CFTR-Cl^- channels localized predominantly in the apical membrane of intestinal crypt cells; and blockade of electroneutral NaCl absorption, most likely through inhibition of the Na^+/H^+ exchanger NHE3 localized in the apical membrane of intestinal villous cells (Figure 3A) (5,42,54). The cystic fibrosis gene encoded CFTR protein functions as the major if not sole cAMP-and cGMP-sensitive Cl^- and HCO_3^- channel in the intestinal epithelium, as is apparent from the complete loss of cAMP- and cGMP-induced intestinal anion secretion in most CF patients and in Cftr null mice (2,55). Among the NHE family members expressed in intestinal epithelium, NHE3 is the major Na^+ importer and the sole isoform known to be inhibited by cAMP-, cGMP- and Ca^{2+} signals (56). Adenoviral overexpression of cGK II in the CFTR-expressing IEC-CF7 intestinal cell line (18,35) and in NHE3-expressing PS120 fibroblasts (51) sensitized CFTR and NHE3 to activation respectively inhibition by cGMP agonists and analogs. In both cell models, membrane anchoring of cGK II through N-terminal myristoylation appeared essential, but, at least in case of NHE3, not sufficient to target the enzyme to its substrate; the additional expression of a tandem PDZ domain scaffolding protein, NHERF2/E3KARP, known to interact with the C-terminus of NHE3 and serving as a low-affinity GKAP, was required for rendering NHE3 cGMP/cGK II-sensitive. Confirmation of this model in vivo awaits the generation and phenotypic characterization of NHERF2 -/- mice.

The intestinal peptides guanylin and uroguanylin, both structurally related to the diarrheogenic *E.coli* heat-stable enterotoxin, are the major if not sole regulators of intracellular cGMP levels and cGK II activity in the enterocytes under physiological conditions (55). They are tonically secreted into the intestinal lumen and interact with the extracellular receptor domain of the heat-stable enterotoxin receptor/guanylyl cyclase C, which is co-localized with cGK II in the apical membrane of the enterocyte. The guanylin/GC-C/cGK II system is thought to prevent dehydration and consequently obstruction of the intestine under physiological conditions but, if hyperactivated in a sustained fashion by microbial enterotoxins, may cause excessive loss of salt and water resulting in secretory diarrhea. Surprisingly, cGK II null mice did not show gross intestinal abnormalities, despite their strong reduction in cGMP-activated intestinal Cl^- secretion and cGMP-inhibition of Na^+ absorption, and their insensitivity to the diarrheogenic effect of heat-stable

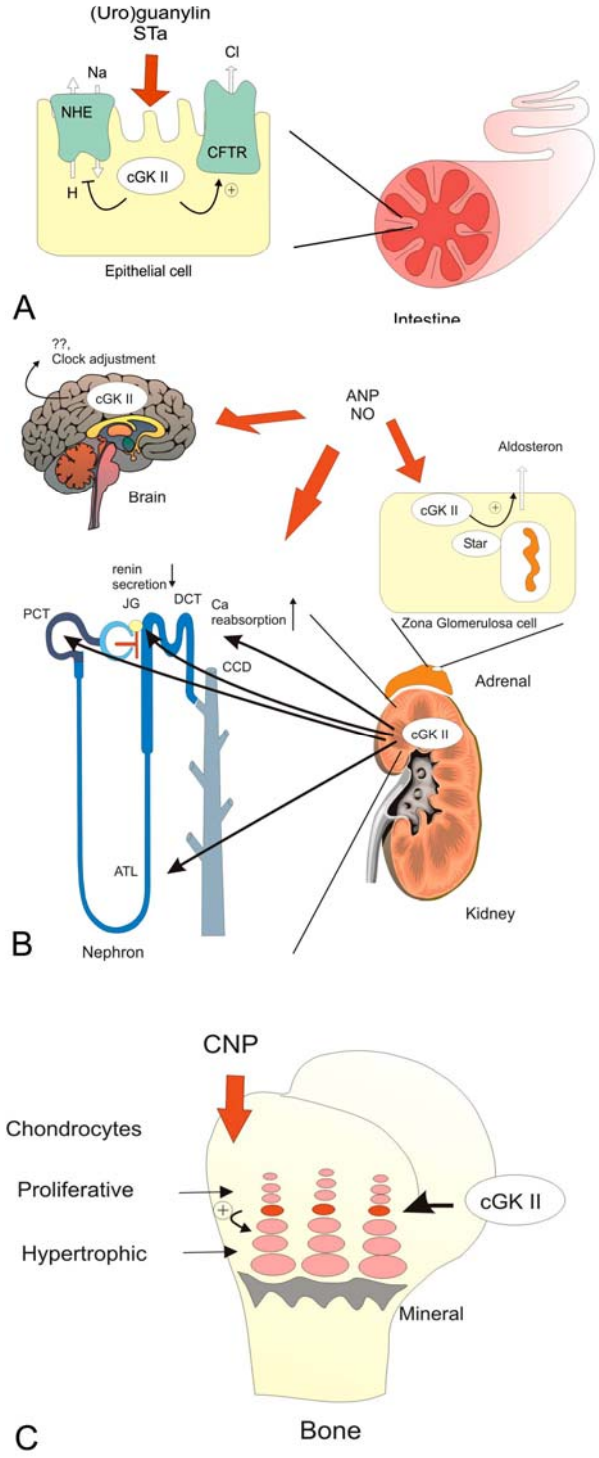

Figure 3. Tissue distribution and various functions of cGK II. Thick red arrows indicate the putative hormones activating cGK II in the various tissues. A, cGK II in the intestine downstream of (uro)guanylin signaling; CFTR, cystic fibrosis transmembrane conductance regulator; STa, heat-stable enterotoxin; NHE, sodium-proton exchanger. B, cGK II in brain, kidney and adrenal downstream of natriuretic peptide and NO signaling. Black arrows indicate the main sites of cGK II expression in the nephron; ATL, ascending thin limb of Henle; CCD, cortical collecting duct; DCT, distal convoluted tubule; JG, juxtaglomerular cell; PCT, proximal convoluted tubule; Star, steroidogenic acute regulatory protein. C, cGK II in bone downstream of C-type natriuretic peptide (CNP) signaling. The red cells indicated by the black arrow represent cGK II expressing chondrocytes in the growth plate.

enterotoxin (5,54). This suggests that the function of cGK II in mouse intestine is either of minor importance for normal physiology, or can be compensated by other systems. Indeed, ion transport in intestinal epithelium is known to be regulated by multiple hormones and auto/paracrinic factors using cAMP and Ca^{2+} as second messengers in addition to cGMP (57); in addition, cGMP may signal independently from cGK II, as it can activate cAK either directly by cross-activation or indirectly by inhibiting type III phosphodiesterase and raising cAMP levels (54).

More recently the guanylin/GC-C/cGK II system has been shown to operate also in other epithelial tissues including distal airways, pancreatic ducts and the gall bladder (10,11,58). As in the intestine, its major function appears to be the luminocrinic regulation of transepithelial electrolyte and water transport, principally at the level of the CFTR-Cl^- channel, which is co-expressed in the same cell type. Localization studies revealed that guanylin and its receptor GC-C are confined exclusively to nonciliated (Clara) cells in the distal conducting airways (10,59), to the centroacinar and proximal duct epithelial cells of the pancreas (58), and to secretory epithelial cells in the gall bladder (11). The ill-functioning of the guanylin/GC-C/cGK II/CFTR pathway for ion and water secretion in cystic fibrosis, by its impact on mucociliary clearance in the lungs and ductular transport of pancreatic enzymes and bicarbonate, may well contribute to the pancreatic and lung disease in CF patients. However, as discussed previously for the intestine, its relative importance in comparison with other CFTR regulatory pathways, in particular the cAMP pathway, is presently unknown. Clearly, a more detailed study of ion and water transport abnormalities in the airways, pancreas and gall bladder of guanylin-, GC-C- and cGK II null mice *in vivo* is needed to further evaluate the functional importance of the guanylin/GC-C/cGK II pathway in these tissues under physiological and pathophysiological conditions.

7.4. Role cGK II in endochondral ossification

One of the most surprising findings in the phenotypic characterization of the cGK $II^{-/-}$ mice was their dwarfism, as a link between skeletal bone formation and cGMP signaling was not anticipated from earlier studies (5). Recently, a natural occurring dwarf rat mutant, the Komeda miniature rat Ishikawa, was found to have a deletion in the cGK II gene (60). From these studies it was established that cGK II, at least in rodents, plays a role in the endochondral ossifications of the long bones and vertebrae but not in the membranous ossifications of flat bones. During endochondrial ossification, chondrocytes, derived from undifferentiated mesenchymal cells, subsequently proliferate, become hypertrophic and synthesise cartilage matrix proteins such as collagen type X. Finally, the terminal hypertrophic chondrocytes stimulate the deposition of calcium phosphate by the production of matrix vesicles before they disappear presumably by apoptosis (61). In normal growth plates, the differentiating chondrocytes are stacked in orderly columns, intersecting the resting, proliferative, hypertrophic and calcifying zones. Because cGK II is expressed mainly in chondrocytes located in the zone between proliferation and hypertrophy a role of cGK II in the conversion of a proliferative to a hypertrophic phenotype may be anticipated (Figure 3C) (5). This suggestion is corroborated by studies on the Komeda miniature rat Ishikawa (60), and on mice over-expressing a constitutively active PTH/PTHrP receptor mutant in chondrocytes (62). The expression of the active PTH receptor resulted in a delay in hypertrophy of the chondrocytes and in a similar histology of the growth plate as observed in cGK $^{-/-}$ mice i.e. an enlarged mixed proliferative-hypertrophic zone in which the regular columnar stacking and the expression of the hypertrophy marker, type X collagen, were disturbed (26,62). The natriuretic peptide CNP, the putative ligand of the receptor guanylyl cyclase GC-B, was shown to contribute to the normal growth of skeletal bones in a cGK II-dependent way, as over-expression of CNP caused longer bones in wild type but not in $cGK^{-/-}$ mice. As expected, targeted deletion of the CNP gene resulted in dwarfism (26). However, other cGMP-stimulating agents may activate cGK II during normal endochondral ossification as well, since the histology of the growth plate of $cGK^{-/-}$ mice differed from that of the $CNP^{-/-}$ mice (26).

The molecular mechanism by which cGK II regulates chondrocyte maturation in mice is largely unknown. It was suggested that cGK II stimulates chondrocyte hypertrophy by inhibiting the nuclear translocation of Sox9, a transcription factor known to prevent differentiation to a hypertrophic cell type (60). On the other hand, targeted over-expression of CNP was shown to inhibit the MAP kinase signaling in the growth plate of achondroplastic mice bearing an activating mutation in the fibroblast growth factor receptor 3, suggesting that cGK II may stimulate chondrocyte hypertrophy by blocking the MAP kinase pathway (63). Interestingly, cGK II may oppose the effect of cAK in chondrocytes. The latter kinase is thought to mediate part of the effects of PTH/PTHrP and promote the proliferative phase and delay further maturation and hypertrophy of the chondrocyte, probably involving activation of the transcription factor CREB (64,65). cGK II was also implicated in the cGMP-induced inhibition of proliferation of stromal prostate cells showing that it may have a role in the regulation of proliferation of other cell types as well (15).

7.5. Role of cGK II in the kidney and adrenal

Depending on its localization within the different (ad)renal cell types, cGK II may have both diuretic and antidiuretic effects (Figure 3B). The best established function of cGK II is the inhibition of renin secretion in juxtaglomerular cells (23,66,67). Renin is the physiological activator of the blood pressure elevating and anti-diuretic hormone angiotensin II. Both in isolated kidneys and in juxtaglomerular cells the stimulation of renin secretion by cAMP-raising hormones and other stimuli was inhibited by cGMP analogs, and this inhibition was abolished in cGK II $^{-/-}$ mice, but not in cGK I null mice (23,66,67). Furthermore, cGK II $^{-/-}$ mice, but not cGK I $^{-/-}$ or wild type mice showed higher levels of renin mRNA under

conditions aimed to stimulate or inhibit the renin-angiotensin system, i.e. high or low salt diet (67). As cGK II was found to be associated with the storage vesicles containing renin, it is ideally located to mediate inhibition of renin secretion (23). These data taken together strongly suggest that, under physiological conditions, cGK II is an essential part of the pathway by which natriuretic peptides and nitric oxide inhibit renin secretion and, therefore, angiotensin II formation and subsequently NaCl and water reabsorption in the kidney. Paradoxically, these cGMP-raising agents may also be capable of stimulating renin secretion via a cAMP-dependent pathway, activated by inhibition of a cGMP-inhibited phosphodiesterase.

Activation of cGK II in other cell types affecting kidney function may result in renal sodium and water retention rather than natriuresis and diuresis. In isolated adrenal zona glomerulosa cells, cGK II was upregulated in response to a low sodium diet, known to inhibit natriuresis (9), and was shown to stimulate the secretion of the anti-diuretic hormone aldosterone, apparently by phosphorylation and activation of the StAR protein (9). In this cell type, similar to the juxtaglomerular cells, a rise in cGMP levels triggered by ANP or NO may not only stimulate basal aldosterone secretion by activating cGK II, but could also inhibit the adrenocorticotropin hormone (ACTH)/cAMP-stimulated secretion of this diuretic hormone by stimulating a type II cGMP-stimulated PDE and thus lowering the concentration of cAMP (68).

cGK II mRNA and protein have also been detected in the epithelial cells of rat proximal tubules, rat ascending thin limb (ATL), and rabbit connecting tubules and cortical collecting ducts (20,23). In response to water deprivation, cGK II expression is upregulated in the ATL, simultaneously with the ClC-K1 channel used for Cl^- reabsorption, suggesting an anti-natriuretic and anti-diuretic action of cGK II in this kidney segment. In contrast, cGK II in the proximal tubule could be involved in ANP/NO/cGMP inhibition of Na^+ reabsorption by the Na^+/H^+ exchanger NHE3, perhaps through a similar mechanism as identified in the intestine (51), contributing to natriuresis rather than promoting Na^+ reabsorption in this segment. Interestingly, disruption of the mouse gene encoding the protein phosphatase 1 inhibitor, DARPP-32, a cGK substrate, caused loss of ANP-induced natriuresis and resulted in increased arterial blood pressure, raising the possibility that cGK might also act indirectly via regulation of protein phosphatase activity (69).

In view of its apparent colocalization with the (uro)guanylin receptor GC-C in various nephron segments (24), it is possible that cGK II contributes to the natriuretic effect of uroguanylin. This hormone was implicated as the intestine-derived signal mediating the enhanced sodium excretion in response to an oral salt load (70). However, the natriuretic effect of uroguanylin was not attenuated in GC-C $^{-/-}$ mice (71), and no changes in blood pressure were reported in cGK $II^{-/-}$ mice, in contrast to uroguanylin null mice (72). These findings suggest that neither GC-C nor cGK II are functionally involved in uroguanylin signalling in the kidney, or, alternatively, that the anti-and pro-diuretic effects of cGK II in the different cell types tend to cancel each other, explaining the lack of an overt renal phenotype in the cGK II null mice. Clearly, microperfusion studies in cGK II $^{-/-}$ mice are needed to identify possible effects of cGK II signalling on ion transport in each nephron segment separately.

In the rabbit, cGK II was found to stimulate the reabsorption of Ca^{2+} by epithelial cells isolated from connecting tubules and cortical collecting ducts (20). The target of cGK II in these cells is likely to be the epithelial Ca channel TRPV5/ECAC1, which contains two consensus phosphorylation sites for cGK II and cAK (Ser^{669} and Thr^{709}). Similar to the CFTR Cl^- channel, the Ca^{2+} uptake could not be stimulated by the non-myristoylated form of cGK II, but unlike CFTR, the membrane targeted form of cGK I beta was unable to stimulate Ca reabsorption (20). This finding suggests that TRPV5 is a relatively poor substrate for cGK I, either because of intrinsic differences in substrate specificity between cGK I and cGK II, or because a sofar unidentified cGK II-specific GKAP assists in targeting the cGK to the channel. The physiological importance of the cGK II-dependent activation of Ca^{2+} reabsorption was speculated to reside in its Ca^{2+} sparing effect during natriuretic peptide-provoked diuresis (20).

7.6. Other functions of cGK II

Despite its abundance in various parts of the brain, as yet no clear functions of cGK II in this organ have been described, except for a role in controlling emotionality and neurobehavioural effects of alcohol (73) and in the resetting of the circadian clock in mice (74). The latter system, controlling various biological rhythms, is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, a region also shown in hamsters and rat to contain cGK II (22,75). From studies with cGK II $^{-/-}$ mice, evidence was provided that cGK II is involved in the signaling pathway by which light delays the circadian clock, presumably by inducing transcription of the Period 2 gene (mPer2) and inhibiting the induction of mPer1 (74). In general the cGK II $^{-/-}$ mice do not suffer from overt neurological disorders, and no effects were observed on long term potentiation in the hippocampus CA1 region of these mice, despite a role for NO-signalling in this process (76). In cultured cells, cGK II was shown capable of inducing gene transcription in neuronal and gliomal cell types (52).

8. PERSPECTIVES

Although cGK II has now gained recognition as a distinctive contributor in cGMP signaling in various tissues, precise knowledge about its function and mechanism of activation lags behind that of the earlier discovered cAKs and cGK I. Especially underdeveloped areas of knowledge, requiring future investigations are:

- the transcriptional regulation of cGK II causing its restricted expression in specific cell types;
- the physiologically relevant substrates of cGK II and anchoring proteins for cGK II required to specifically interact with these substrates;

- the function of cGK II in the various regions of the brain. Regarding the role of cGKs in the nervous system, it may be of relevance that in invertebrates like fruit flies or honey bees, an isoform of cGK was found to be important for certain kinds of behaviors like foraging (77,78);

- the role of cGK II as a regulator of cell proliferation and apoptosis. Several lines of evidence support a link between cGMP signalling and cell growth: First, as discussed previously, cGK II may stimulate chondrocyte hypertrophy by blocking the MAP kinase pathway (63) and inhibit proliferation of human prostatic stromal cells (15). Secondly, the other isoenyme, cGK I, can have pro-or antiproliferative, and pro-or antiapoptotic effects, dependent on the cell type, and mediated at least in part through its effect on MAP kinase pathways (79). Thirdly, uroguanylin treatment suppressed polyp formation in the $Apc^{Min/+}$ mouse model of colorectal cancer, and uroguanylin, heat-stable enterotoxin and 8-Br-cGMP were shown to inhibit cell proliferation and to induce apoptosis in the T84 human colon carcinoma cell line via cGMP (80,81). However, in the latter case the activation of CNG channels, rather than activation of cGK II has been implicated as the most plausible mechanism underlying the antiproliferative effect of cGMP (80). Finally, in unicellular organisms, e.g. amoebae, cGMP signaling is mediated by other forms of high-affinity cGKs, some of them harbouring additional functional domains involved in a network of Ras signaling, including a Ras, Ras-GEF and MEK kinase domain (82);

- Identification of other transporters aside CFTR, NHE3 and TRPV5/ECAC1 that are regulated by cGK II. Because of the preferential location of the lipidated enzyme in the plasma membrane, cGK II is ideally suited to act as a transport regulator. Candidate transporters include epithelial Na^+ channels (ENaC), apical K^+ channels and transient receptor potential (TRP) channels. In distal lung epithelial cells, ENaC channels are inhibited by NO donors, guanylin, and PDE5 inhibitors, possibly involving cGK II (83,84); though still speculative, this action may provide the rationale for the recent observation that the hyperacidification defect of the *trans*-Golgi network in cystic fibrosis airway epithelial cells can be corrected by GMP agonists (84,85).

Finally, to better study the function of cGK II under physiological conditions, and because of the obvious clinical benefit in the treatment of diarrheal diseases, the development of specific and potent inhibitors of cGK II would be a worthwhile endeavour.

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Abbreviations: 8-pCPT-cGMP: 8-(4-chlorophenylthio)-cGMP, PET-cGMP: β-phenyl-1,N^2-etheno-cGMP, 8-Br-PET-cGMP: β-phenyl-1,N^2-etheno-8-bromo-cGMP, Rp-8-pCPT-cGMPS: Rp isomer of 8-(4-chlorophenylthio)-guanosine-3'-5'-cyclic monophosphoro-thioate, Rp-8-Br-PET-cGMPS: Rp isomer of β-phenyl-1,N^2-etheno-8-bromo-guanosine-3'-5'-cyclic monophosphorothioate, cAK: cAMP-dependent protein kinase, CFTR, cystic fibrosis transmembrane conductance regulator, cGK: cGMP-dependent protein kinase, CNG: cyclic nucleotide gated channel, CNP: C-type natriuretic peptide, ECaC: epithelial Ca^{2+} channel, ENaC: epithelial Na^{+} channels, GC: guanylyl cyclase, GEF: GTP exchange factor, GKAP: cGK anchoring protein, MAP kinase: mitogen-activated protein kinase, MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, PDE: phosphodiesterase, PTH: parathyroid hormone, PTHrP: parathyroid hormone related protein, PTPS: 6-pyruvoyltetrahydropterin synthase, StAR: Steroidogenic acute regulatory protein, TRP: transient receptor potential

Key Words: cyclic GMP, cGMP kinase, Cyclic Nucleotides, Protein Kinases, Epithelial Ion Transport, Natriuresis, Chondrogenesis, Diarrheal Disease, Cystic fibrosis, Review

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