

# Endogenous Type II cGMP-dependent Protein Kinase Exists as a Dimer in Membranes and Can Be Functionally Distinguished from the Type I Isoforms\*

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In mammalian tissues two types of cGMP-dependent protein kinase (cGK) have been identified. In contrast to the dimeric cGK I, cGK II purified from pig intestine was shown previously to behave as a monomer. However, recombinant rat cGK II was found to have hydrodynamic parameters indicative of a homodimer. Chemical cross-linking studies showed that pig cGK II in intestinal membranes has a dimeric structure as well. However, after purification, cGK II was found to be partly proteolyzed into C-terminal monomeric fragments. Phosphorylation studies in rat intestinal brush borders revealed that the potency of cGMP analogs to stimulate or inhibit native cGK II *in vitro* (i.e. 8-(4-chlorophenylthio)-cGMP > cGMP >  $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-8-bromo-cGMP >  $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-cGMP and  $R_p$ -8-(4-chlorophenylthio)-cGMPs >  $R_p$ - $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-8-bromo-cGMPs, respectively) correlated well with their potency to stimulate or inhibit cGK II-mediated Cl<sup>-</sup> secretion across intestinal epithelium but differed strikingly from their potency to affect cGK I activity. These data show that the N terminus of cGK II is involved in dimerization and that endogenous cGK II displays a distinct activation/inhibition profile with respect to cGMP analogs, which permits a pharmacological dissection between cGK II- and cGK I-mediated physiological processes.

Cyclic GMP-dependent protein kinases (cGKs)<sup>1</sup> play an important role in the signaling of various cGMP-linked hormones and neurotransmitters including nitric oxide (NO), natriuretic peptides, and guanylin (1, 2). In mammalian tissues two types of cGK have been identified. Type I cGK, consisting of  $\alpha$  and  $\beta$  isoforms, is more generally expressed and acts as a key regulator of cardiovascular homeostasis (1, 2). In contrast, type II

cGK was described originally as an intestine-specific form (3). Molecular cloning demonstrated that cGK II is indeed a distinct gene product expressed predominantly in epithelial cells of the intestine (4), although its mRNA was also detected in kidney, bone, and brain (4–7). Its widespread distribution in various areas of the brain suggests an important role of cGK II in NO/cGMP signaling in the central nervous system (6).

In the intestine, cGMP is involved in the regulation of ion and water transport. It inhibits the uptake of NaCl and stimulates the secretion of Cl<sup>-</sup> by activating the cystic fibrosis trans-membrane conductance regulator, a Cl<sup>-</sup> channel that is mutated in CF patients (8, 9). Guanylin, a small peptide derived from a larger precursor protein released lumenally by intestinal epithelial cells, may function as the physiological activator of the cGMP-mediated signaling pathway in intestine by activating guanylyl cyclase C (10, 11). Heat-stable enterotoxins secreted by various pathogenic strains of *Escherichia coli* mimic the action of guanylin and elicit a severe secretory diarrhea by hyperactivating guanylyl cyclase C (8, 9). Electrophysiological and immunolocalization studies have provided evidence for a key role for cGK II as a mediator of the cGMP-provoked intestinal Cl<sup>-</sup> secretion (12–14). The critical role of cGK II in STa/cGMP-induced diarrhea was recently confirmed by pharmacological and gene disruption techniques (7, 15).

Sequence comparison and biochemical analysis revealed a large degree of similarity in the structural organization of cGK I and II (2–5). Both isotypes possess two cGMP binding domains on one polypeptide chain which is covalently coupled to a catalytic domain. Their N terminus contains an autoinhibitory region, one or more autophosphorylation sites, and a leucine zipper motif, and both proteins are devoid of hydrophobic trans-membrane domains. Despite these similarities, cGK II was shown to differ from soluble dimeric type I cGK in that it behaved as a membrane- and cytoskeleton-associated protein in intestinal brush borders and as a monomer following its solubilization and purification (3). However, recombinant mouse brain cGK II was reported to be soluble and dimeric after expression in mammalian and insect cells (5, 16). In contrast, recombinant rat intestinal cGK II was found to be tightly bound to the membrane after expression in similar cell systems (4, 17, 18), but its oligomerization state was not established. Furthermore, the  $K_a$  values for cGMP and cGMP analogs of recombinant rat intestine cGK II purified from Sf9 cells (17) differed considerably from those of recombinant mouse brain cGK II, which contained an additional N-terminal histidine tag (16).

Since rat and mouse cGK II have very similar sequences, are endogenously both membrane-bound in intestine, and are ac-

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<sup>1</sup> The abbreviations used are: cGK, cGMP-dependent protein kinase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; PET-cGMP,  $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-cGMP; 8-Br-PET-cGMP,  $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-8-bromo-cGMP;  $R_p$ -8-pCPT-cGMPs,  $R_p$  isomer of 8-(4-chlorophenylthio)-guanosine-3'-5'-cyclic monophosphorothioate;  $R_p$ -8-Br-PET-cGMPs,  $R_p$  isomer of  $\beta$ -phenyl-1, *N*<sup>2</sup>-etheno-8-bromo-guanosine-3'-5'-cyclic monophosphorothioate; I<sub>SC</sub>, short-circuit current; PAGE, polyacrylamide gel electrophoresis.

tivable by similar concentrations of cGMP after expression in COS-1 cells (5, 18), the observed kinetic and structural differences among purified cGK II preparations may reflect artifacts of the expression system and/or purification rather than fundamental differences. To circumvent such potential artifacts, we characterized the oligomerization state of cGK II and its kinetic properties in an environment most relevant for its physiological functioning, *i.e.* intestinal brush border membranes. We also compared the stimulatory or inhibitory potency of various cGMP analogs toward cGK II *in vitro*, with their effects on Cl<sup>-</sup> secretion in rat intestinal epithelium, presumably a cGK II-mediated process (7, 9, 12–15).

We report here that the endogenous intestinal membrane-bound type II cGMP-dependent protein kinase exists as a dimer and displays a distinct activation profile with respect to cGMP analogs, which permits discrimination between cGK II and cGK I effects in physiological processes.

#### EXPERIMENTAL PROCEDURES

**Materials**—Disuccinimidyl suberate was obtained from Pierce, and 3-isobutyl-1-methylxanthine was from Sigma. Polyclonal cGK II or cGK I antibodies, raised against recombinant cGK II or cGK I $\beta$  expressed in *E. coli*, were prepared as described (12). The cGK substrate peptide 2A3 (RRKVSQKE) and the Walsh inhibitor peptide (protein kinase A inhibitor-(5–24)-amide) were synthesized by D. Palm (University of Würzburg, Germany), and the cGMP analogs were from Biolog (Bremen). cGK I (primarily the I $\alpha$  isoform; Ref. 13) was purified from bovine lung as described (19).

**Preparation of Various cGK II-containing Membranes and Purified cGK II**—Confluent HEK-293 or NIH-3T3 cells, stably transfected with cGK II (18), were washed twice with ice-cold phosphate-buffered saline, scraped with a rubber policeman in buffer A (150 mM NaCl, 10 mM NaPO<sub>4</sub>, pH 7.4, 1 mM EDTA, 100  $\mu$ g/ml trypsin inhibitor, and 20  $\mu$ g/ml leupeptin) and processed directly or frozen in liquid N<sub>2</sub> and stored at -80 °C. The cells were homogenized by brief sonication (three bursts of 3 s, peak-to-peak amplitude 15–20  $\mu$ m), and a crude membrane fraction was prepared by centrifugation for 15 min at 20,000  $\times$  *g*. Membranes from HEK-cGK II cells were resuspended in buffer A (2–3 mg of protein/ml), and membranes from 3T3-cGK II cells (which had lower basal protein kinase activity in cGMP activation assays) were resuspended to 1 mg of protein/ml in buffer B (20 mM Tris/HCl, pH 7.4, 5 mM  $\beta$ -mercaptoethanol, 2 mM EDTA, 100  $\mu$ g/ml trypsin inhibitor, and 20  $\mu$ g/ml leupeptin).

Non-vesiculated brush border caps were prepared from jejunum and ileum of male Wistar rats by vibration of everted intestine in hypotonic EDTA buffer and low speed centrifugation, essentially as described previously (20), and were finally resuspended to 0.4 mg of protein/ml in buffer B.

Brush border membrane vesicles were isolated from everted pig intestine by freeze-thawing, followed by Mg<sup>2+</sup> precipitation according to Ref. 21. cGK II was solubilized from HEK-cGK II membranes or pig brush border membrane vesicles by addition of 0.5 M NaCl and 1% Triton X-100 and subsequently purified by affinity chromatography on 8-(2-aminoethyl)-amino-cAMP-Sepharose as described (3).

**Gel Filtration and Sucrose Density Gradient Centrifugation**—Gel filtration was performed essentially as described (22) using Superose 6 HR 10/30 or Superdex 200 HR 10/30 analytical gel filtration columns (Pharmacia Biotech Inc.) equilibrated and subsequently eluted at 0.2 ml/min with 500 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4, 10 mM EDTA, and 0.1% Triton X-100. Fractions of 400  $\mu$ l were collected and analyzed by immunoblotting or protein kinase activity assays. For each experiment the column was calibrated using standards.

Sucrose density centrifugation was performed with linear 7.5–25% sucrose gradients in the same buffer used for gel filtration (22). Apparent sedimentation coefficients ( $s_{w,20}$ ) were calculated by plotting the distance from the top of the gradient against the position of the following standards: catalase (11.3 S), bovine serum albumin (4.9 S), and cytochrome *c* (1.9 S).

The molecular mass (*m*) of cGK I and cGK II was calculated from:  $m = 6\pi\eta N a s_{w,20} / (1 - \nu\rho)$  where  $\eta$  = viscosity of medium,  $n$  = Avogadro's number,  $a$  = Stokes radius,  $s_{w,20}$  = sedimentation coefficient,  $\nu$  = partial specific volume, and  $\rho$  = density of the medium. We assumed  $\nu$  for proteins to be 0.73 cm<sup>3</sup>/g (22).

**Protein Sequencing and Cross-linking of cGK II**—Purified pig cGK II was separated by SDS-PAGE and blotted to polyvinylidene difluoride

Problott<sup>®</sup> membrane. Pieces of membrane containing the 75- and 70-kDa forms of cGK II were cut out separately, and both protein fragments were N-terminally sequenced by automatic Edman degradation with a 473A protein sequencer (Applied Biosystems).

For cross-linking, samples (2 mg of protein/ml) were incubated for 15 min at 0 °C in 10 mM phosphate buffer, pH 7.4, 100 mM NaCl, 10 mM EDTA with or without 0.6 mM disuccinimidyl suberate. The reaction was stopped by addition of SDS-PAGE sample buffer and cGK II was analyzed by immunoblotting.

**Immunoblotting and Protein Kinase Assays**—Immunoblotting was performed as described earlier (23). Immunoreactive proteins were detected after incubation with cGK II or cGK I antibody (1:3000) by the enhanced chemiluminescence method (Amersham Corp.) and quantitated by densitometric scanning (Bio-Rad, model 620).

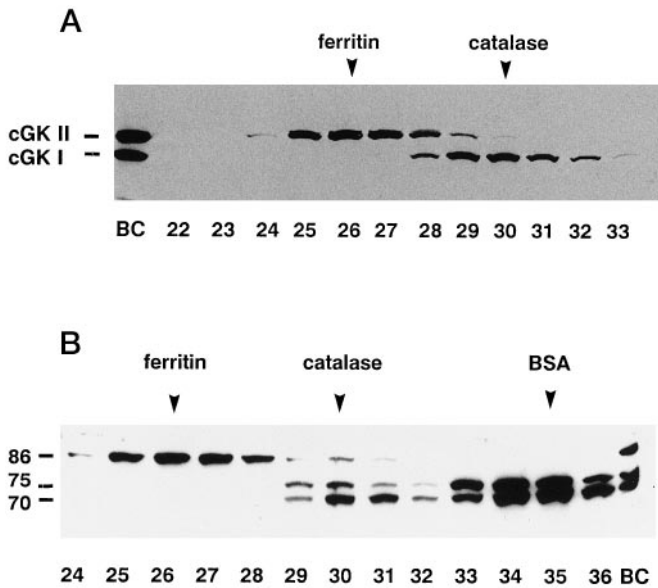
Protein kinase activity was determined by incubation of the samples (4–10  $\mu$ g of membrane protein in case of cGK II or 30 ng of purified cGK I provided with 10  $\mu$ g of bovine serum albumin) at 30 °C for different times in 40  $\mu$ l of 20 mM Tris/HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 5 mM  $\beta$ -mercaptoethanol, 0.1 mM 3-isobutyl-1-methylxanthine, 25 mM sodium  $\beta$ -glycerophosphate, 200 nM protein kinase A inhibitor, 0.1 mg/ml cGK substrate peptide 2A3 (RRKVSQKE; Ref. 17), 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and various concentrations of nonradioactive Mg-ATP and cGMP or cGMP analogs as indicated. The reaction was started by addition of 10- $\mu$ l aliquots of the cGK II preparations to 30  $\mu$ l of prewarmed incubation buffer and quenched by addition of 10  $\mu$ l of 0.5 M EDTA. The samples were subsequently centrifuged for 10 min at 20,000  $\times$  *g*. The pellet fraction was resuspended in SDS-PAGE sample buffer for determination of the autophosphorylation of cGK II (3), and 15  $\mu$ l of the supernatant was spotted in duplicate on sheets of P-81 chromatography paper (Whatman). After 4 washes with 1% phosphoric acid the amount of label incorporated was quantitated with the Molecular Imaging System GS-363 (Bio-Rad). The phosphotransferase activity of cGK II as measured in intestinal brush border and 3T3 cell membranes and the activity of cGK I were linear with the amount of enzyme up to the concentration used in the kinetic study and with time up to 4 min in the presence of relatively high ATP concentrations ( $\geq$ 300  $\mu$ M). However, in the presence of low ATP, the cGK II kinase activity started to deviate from linearity after 2 min. This nonlinearity was independent of the concentration of cGMP or cGMP analogs and apparently resulted from ATP depletion caused by the action of endogenous ATPases (data not shown). The  $K_m$  of cGK II for ATP was therefore determined from 2-min incubations, whereas the kinetic parameters for the cGMP analogs were routinely determined on the basis of 4-min incubations. The latter condition resulted in similar kinetic values but higher signal to noise ratios in comparison with 2-min incubations.

**Electrical Measurements in Rat Intestinal Mucosa**—A 1-cm long segment of rat cecum was removed under light diethyl ether anesthesia. The muscle layers were stripped off by blunt dissection, and the mucosa was mounted in an Ussing chamber (0.3-cm<sup>2</sup> area exposed) for measurements of short-circuit current (I<sub>sc</sub>) across the tissue as described (24). Dose-response curves were obtained by cumulative additions of agonists or antagonists, and subsequent measurements of the plateau phase of the I<sub>sc</sub> reached 10–20 min after each addition.

**Calculation of Kinetic Parameters and Determination of Lipophilicity of cGMP Analogs**—The values for EC<sub>50</sub>, IC<sub>50</sub>, and apparent  $K_i$  (defined as the concentration required for half-maximal activation) were determined from dose-response curves fitted by the program Slidewrite.  $K_m$  and Hill coefficients were calculated with the program Enzfitter.  $K_i$  values were determined from Dixon plots at half-maximal concentration of agonist (cGMP), assuming a single site. The lipophilicities of cGMP analogs were determined by gradient reversed phase chromatography essentially as described (25) and comparable to common log *p* values.

#### RESULTS

**Oligomeric State of Recombinant Rat Intestine and Endogenous Pig Intestine cGK II**—Immunoblots demonstrated that recombinant rat cGK II solubilized from HEK 293 membranes was eluted from gel filtration as a single peak with a Stokes radius of 6.6 which is significantly larger than that of purified cGK I $\alpha$  (5.1) eluted under the same conditions (Fig. 1A and Table I). Velocity sedimentation analysis likewise revealed single peaks for cGK II and I $\alpha$  with sedimentation coefficients of 6.8 and 7.2 S, respectively (Table I). From these hydrodynamic parameters the molecular mass of cGK II was calculated as 190 kDa, more than twice the monomeric mass of 87 kDa (4),



**FIG. 1. Gel filtration profiles of crude recombinant and purified endogenous cGK II.** *A*, solubilized membrane proteins from HEK 293 cells stably expressing rat intestine cGK II were mixed with purified bovine cGK I and applied to a Superdex 200 HR gel filtration column. After elution, the presence of cGK was monitored in each fraction by immunoblotting using specific antibodies against cGK II and cGK I. *B*, purified pig intestine cGK II was subjected to gel filtration, and the cGK II content of the fractions eluted was determined by immunoblotting using a specific antibody against cGK II. On the left the molecular masses of the immunoreactive forms of cGK II are indicated in kDa. Fraction numbers are indicated below the panels. *BC* denotes the original preparation before it was injected on the column. The elution positions of standards are indicated for ferritin (Stokes radius,  $a = 6.1$  nm), catalase ( $a = 5.2$  nm), and bovine serum albumin (*BSA*) ( $a = 3.5$  nm).

indicating that cGK II most likely exists as a homodimer in solution. cGK II immunoprecipitation or purification by cAMP-Sepharose chromatography did not reveal any associated proteins that could falsify the mass determination (data not shown, Ref. 18).

In previous studies cGK II purified from pig intestine was found to behave as a monomer in gel filtration and sucrose density sedimentation analyses, although kinase activity, not immunoblotting as above, was used for cGK II detection (3). We therefore compared the hydrodynamic parameters of recombinant rat cGK II with those of native pig cGK II after purification. As shown in Table I, purification had no effect on the dimeric state of recombinant rat cGK II. However, purified pig cGK II was recovered in two major peaks after gel filtration as detected by immunoblotting (see Fig. 1*B*). In the first peak, full-length cGK II (86 kDa) eluted at a position indicative of a dimer. Subsequently, 75- and 70-kDa cGK II fragments eluted at a position corresponding to a Stokes radius of 3.5 nm, similar to that found for monomeric cGK II previously (3). In accordance with the previous study, most of the kinase activity (>90%) was recovered in the second peak (data not shown). The proteins in the second peak were identified by protein sequencing as C-terminal, proteolytic fragments of cGK II. The N-terminal sequences VPLDV and PPEF obtained from the 75- and the 70-kDa form, respectively, matched those of a 75-kDa cGK II fragment beginning at Val<sup>101</sup> and of a 70-kDa fragment starting at Pro<sup>139</sup> (4).

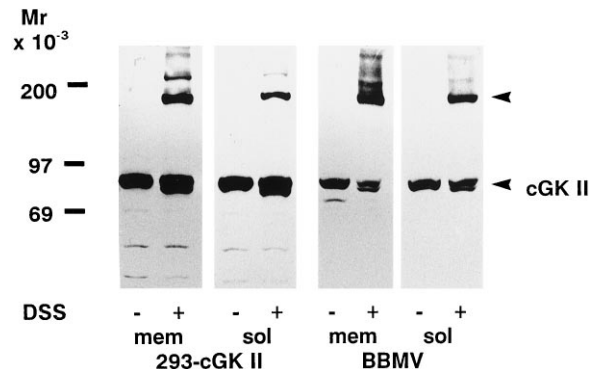
To exclude the possibility that cGK II dimerization could be an artifact of the solubilization process, we compared the oligomeric structure of cGK II in membranes with that of solubilized cGK II by chemical cross-linking. As shown in Fig. 2, addition of the cross-linker disuccinimidyl suberate to pig

TABLE I

## Hydrodynamic parameters of recombinant and endogenous cGK II

The Stokes radius was determined by gel filtration; the sedimentation coefficient by sucrose density centrifugation, and the molecular weight was calculated from these parameters as described under "Experimental Procedures." Recombinant cGK II was analyzed either directly after solubilization of membranes from HEK-293 cells stably transfected with rat cGK II (Recomb. cGK II) or after subsequent purification (recomb. cGK II pure). cGK II purified from pig intestine was found to consist of a 86-kDa full-length form and two fragments of approximately 75 and 70 kDa (see Fig. 1). Purified bovine lung cGK I (consisting mainly of the type 1 $\alpha$  form) was added to the recombinant cGK II preparations as an internal standard (Stokes radius = 5.0–5.3 nm, apparent  $s_{20,w} = 6.9$ –7.8 S and  $M_r = 150,000$ –178,000; Refs. 3, 13, and 27). Data represent means  $\pm$  S.E. of three experiments, ND, not determined.

Sample	Stokes radius	Apparent $s_{20,w}$	$M_r \times 10^{-3}$ calculated
	nm	S	
Recomb. cGK II (rat)	$6.6 \pm 0.2$	$6.8 \pm 0.3$	190
Recomb. cGK II pure	$6.4 \pm 0.2$	$7.0 \pm 0.2$	190
cGK II pure (pig intestine)			
86-kDa form	$6.3 \pm 0.2$	ND	
75–70-kDa forms	$3.5 \pm 0.1$	ND	
cGK I pure (bovine lung)	$5.1 \pm 0.2$	$7.2 \pm 0.4$	157



**FIG. 2. Cross-link pattern of recombinant and endogenous cGK II.** Membranes from HEK 293 cells stably expressing rat cGK II (293-cGK II) and pig brush border membrane vesicles (*BBMV*) were incubated either directly (*mem*) or after solubilization with 0.5 M NaCl and 1% Triton X-100 (*sol*) in the presence or absence of 0.6 mM disuccinimidyl suberate (*DSS*). After separation on 7.5% SDS-PAGE, cGK II was detected by immunoblotting. Arrowheads indicate the positions of the 86-kDa monomeric and 170-kDa dimeric forms of cGK II.

brush border membrane vesicles or to membranes of HEK cells stably transfected with recombinant cGK II, either before or after solubilization, resulted in the appearance of a cGK II complex at the position of a dimer (170 kDa) in SDS-PAGE in all cases, indicating that cGK II is also dimeric in membranes. The additional cross-linked bands observed at 80- and 210-kDa positions may represent intrachain or multiple interchain cross-linked forms of cGK II.

**Characterization of cGK II Activity in Rat Intestinal Brush Border Membranes**—Non-vesiculated brush border caps freshly isolated from rat intestine were found to offer a suitable model for measuring cGK II activity in its native membrane environment. They are enriched in cGK II (0.5–1  $\mu$ g of kinase/mg of protein; Refs. 3, 21), contain no detectable levels of cGK I (data not shown, cf. Refs. 3, 9, and 12), and are fully accessible to ATP and exogenous peptide substrates (21). Furthermore, cGMP could stimulate the phosphorylation of exogenous substrate by brush border preparations to a similar extent (7-fold) as reported for purified recombinant cGK II (Fig. 3; Refs. 16 and 17). The apparent  $K_m$  of membrane-bound cGK II for the substrate peptide 2A3 was found to be similar to that of cGK I (0.10 and 0.12 mM, respectively; data not shown).

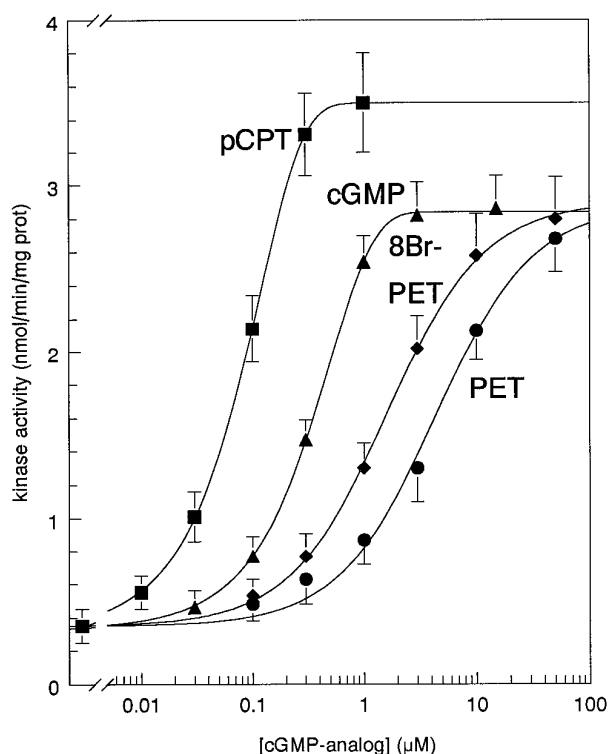


FIG. 3. Stimulation of protein kinase activity in rat intestinal brush border caps by cGMP and cGMP analogs. Phosphorylation of 2A3 by rat intestinal brush border caps was measured in the presence of 300  $\mu\text{M}$  ATP and various concentrations of cGMP ( $\blacktriangle$ ), 8-pCPT-cGMP ( $\blacksquare$ ), 8-Br-PET-cGMP ( $\blacklozenge$ ), or PET-cGMP ( $\bullet$ ). Data are means  $\pm$  S.E. of three experiments.

However, endogenous cGK II at saturating cGMP (10  $\mu\text{M}$ ) has a relatively high  $K_m$  for ATP (0.40 mM) in comparison to cGK I (0.066 mM) (see Fig. 4A). Moreover, ATP was observed to decrease the sensitivity of cGK II for cGMP. As shown in Fig. 4B, the apparent  $K_a$  for cGMP shifted more than 10-fold, from 50 nM at 10  $\mu\text{M}$  ATP to 560 nM at 1 mM ATP. A similar shift in the apparent  $K_a$  for cGMP by ATP in the range of 10–100  $\mu\text{M}$  was noticed in case of cGK I. In the following experiments we routinely determined cGK kinase activity in the presence of 300  $\mu\text{M}$  ATP, since this condition ensured a relatively high signal-to-noise ratio and was comparable with that used in similar studies characterizing purified cGK II (16, 17). At 300  $\mu\text{M}$  ATP using 2A3 as substrate, the apparent  $K_a$  for cGMP of endogenous cGK II was found to be almost identical to that of purified bovine lung cGK I (0.36 and 0.38  $\mu\text{M}$ , respectively; Fig. 4B and Table II). Using the same conditions, a similar apparent  $K_a$  for cGMP (0.31  $\pm$  0.03  $\mu\text{M}$ ;  $n = 3$ ) was found for recombinant membrane-bound cGK II expressed in NIH 3T3 cells (not shown), indicating that the sensitivity of recombinant rat cGK II for cGMP is similar to that of endogenous rat cGK II and is not critically dependent on its native environment. However, solubilization of recombinant cGK II with Triton X-100 and 0.5 M NaCl caused a small increase (1.6  $\pm$  0.2-fold;  $n = 3$ ) in its apparent  $K_a$  for cGMP (not shown). We determined the  $K_a$  for cGMP in solubilized 3T3-cGK II membranes, because cGK II in solubilized rat intestinal brush borders was found to be partially converted into 75–70-kDa proteolytic fragments during protein kinase assays (data not shown), similar to pig cGK II during purification (Fig. 1B).

To further characterize native cGK II, we determined the apparent  $K_a$  for various cGMP analogs. The membrane-permeant analog 8-pCPT-cGMP appeared 4–5 times more potent than cGMP in activating cGK II, whereas PET-cGMP and 8-Br-PET-cGMP, in comparison with cGMP, were relatively

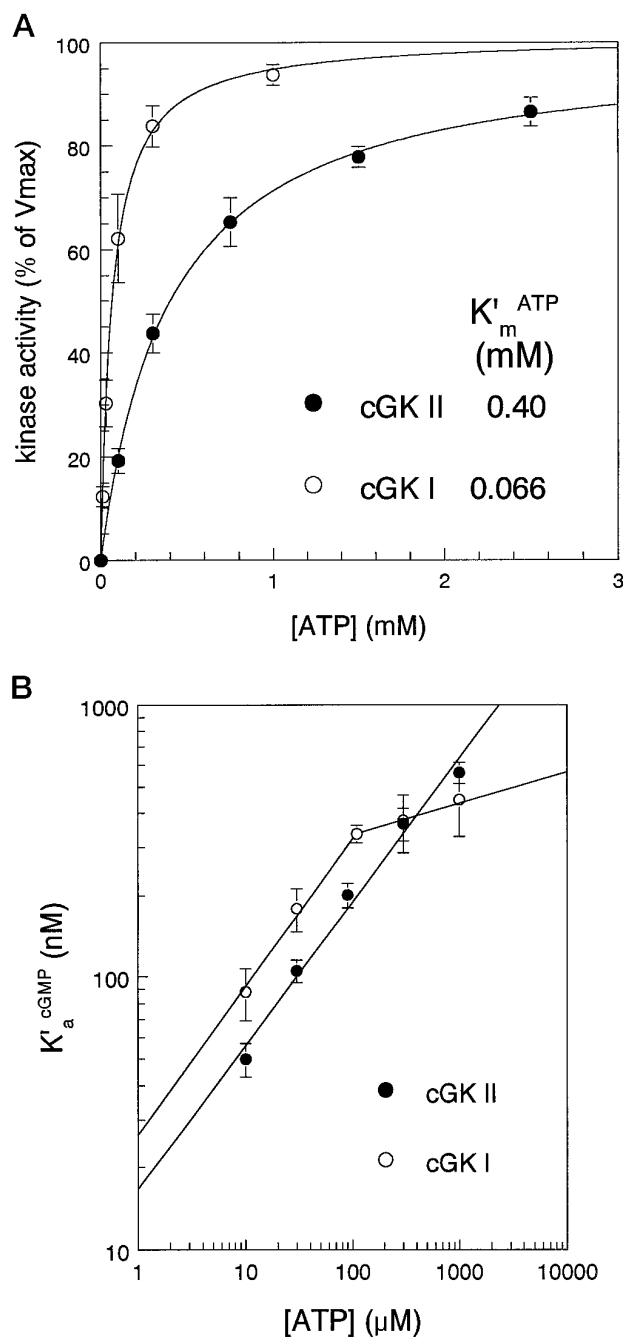


FIG. 4. Kinetic properties of cGK II- and cGK I-catalyzed 2A3 phosphorylation. A, phosphotransferase activity of cGK II in rat intestinal brush border caps ( $\bullet$ ) and of purified bovine lung cGK I ( $\circ$ ) plotted at various concentrations of ATP in the presence of 10  $\mu\text{M}$  cGMP. Data are expressed as percentage of the  $V_{\text{max}}$  values, which were  $5 \pm 1$  nmol/min/mg protein and  $2.7 \pm 0.5$   $\mu\text{mol}/\text{min}/\text{mg}$  protein for cGK II and cGK I, respectively. B, the apparent  $K_a$  for cGMP of the phosphotransferase activity of cGK II in rat intestinal brush border caps ( $\bullet$ ) and of purified bovine lung cGK I ( $\circ$ ) measured at various concentrations of ATP. Data are means  $\pm$  S.E. of three experiments.

poor agonists, showing 12- and 5-fold higher apparent  $K_a$  values, respectively (Fig. 3 and Table II). Both cGMP and 8-pCPT-cGMP, but not the two PET-cGMP derivatives, showed cooperative kinetics, as apparent from their Hill coefficient of  $1.5 \pm 0.2$  ( $n = 3$  for both; not shown).

In addition to stimulating substrate phosphorylation, cGMP was shown previously to increase the autophosphorylation of cGK II in brush border membranes (3). To establish whether both processes have similar kinetic characteristics, we com-

TABLE II

Comparison of the potencies of cGMP analogs to stimulate or inhibit phosphorylation of 2A3 and auto-phosphorylation in rat intestinal brush border caps with their effects on short circuit currents in rat cecum

Autophosphorylation of cGK II or phosphorylation of 2A3 was measured by incubation of brush border caps in the presence of 5 or 300  $\mu\text{M}$  ATP, respectively, and various concentrations of cGMP or the cGMP analogs indicated.  $\text{IC}_{50}$  values for the  $R_p$  analogs on the 2A3 phosphorylation were determined in the additional presence of 0.3 or 3  $\mu\text{M}$  cGMP. Changes in short circuit current ( $I_{sc}$ ) were measured in stripped rat cecum in an Ussing chamber after cumulative additions of permeable cGMP analogs to the serosal side.  $\text{IC}_{50}$  values for the  $R_p$  analogs were determined after stimulation with 2.5  $\mu\text{M}$  8-pCPT-cGMP (cf. Fig. 6). Kinetic parameters were derived from dose-response plots as described under "Experimental Procedures" (cf. Figs. 3 and 5) and are means  $\pm$  S.E. of at least three experiments. ND, not determined.

cGMP analog	Phosphorylation, apparent $K_a$		$\frac{\Delta I_{sc}}{EC_{50}}$	Lipophilicity	
	2A3, 300 $\mu\text{M}$ ATP <sup>a</sup>	Auto, 5 $\mu\text{M}$ ATP <sup>a</sup>		log $K_w$	Relative lipophilicity
	$\mu\text{M}$		$\mu\text{M}$		
cGMP	0.36 $\pm$ 0.09	0.005	ND	0.77	1
PET-cGMP	4.2 $\pm$ 1.5	0.2	850 $\pm$ 300	2.47	50
8-pCPT-cGMP	0.08 $\pm$ 0.01	0.0005	4 $\pm$ 1	2.52	56
8-Br-PET-cGMP	1.6 $\pm$ 0.4	ND	200 $\pm$ 40	2.83	115
	$\text{IC}_{50}^b$		$\text{IC}_{50}$		
	0.3 $\mu\text{M}$ cGMP <sup>c</sup>	3 $\mu\text{M}$ cGMP <sup>c</sup>	2.5 $\mu\text{M}$ pCPT-cGMP <sup>c</sup>		
	$\mu\text{M}$		$\mu\text{M}$		
$R_p$ -8-pCPT-cGMPS	0.29 $\pm$ 0.06	5.4 $\pm$ 0.7	14 $\pm$ 4	2.6	68
$R_p$ -8-Br-PET-cGMPS	0.9 $\pm$ 0.3	13.4 $\pm$ 4.1	14 $\pm$ 5	2.83	115

<sup>a</sup> ATP concentrations chosen are close to the  $K_m$  for ATP of each phosphorylation reaction.

<sup>b</sup> Based on 2A3 phosphorylation only.

<sup>c</sup> Concentration of agonist.

pared their ATP and cGMP analog sensitivity. Surprisingly, under the same assay conditions the  $K_m$  for ATP of cGK II autophosphorylation was 50-fold lower (5–10  $\mu\text{M}$ ) than for 2A3 phosphorylation (data not shown). We therefore determined apparent  $K_a$  values for cGMP and cGMP analogs for cGK II autophosphorylation at low concentrations of ATP (5  $\mu\text{M}$ ). As shown in Table II, there is a close correlation between the relative potency of the cGMP analogs to stimulate auto- and substrate phosphorylation, although 2A3 phosphorylation required 5–10-fold higher concentrations of cGMP or 8-pCPT-cGMP than self-phosphorylation (in the presence of 2A3) when determined at the same (low) concentration of ATP (data not shown; cf. Table II and Fig. 4B).

Another possible difference between autophosphorylation and substrate phosphorylation by cGK II may be their response to  $R_p$ -diastereoisomers of phosphorothioate-modified cGMP analogs. These compounds were previously shown to stimulate autophosphorylation of cGK II in rat intestinal brush border membranes (9) but to inhibit substrate phosphorylation by purified recombinant cGK I and II (16, 17). To exclude the possibility that the different responses to  $R_p$ -cGMPS analogs were caused by differences in the origin/environment of cGK II (purified recombinant *versus* membrane-bound endogenous), we tested these analogs on cGK II activity in brush border membranes. As shown in Fig. 5 and Table II, both  $R_p$ -8-pCPT-cGMPS and  $R_p$ -8-Br-PET-cGMPS inhibited 2A3 phosphorylation by endogenous membrane-bound cGK II in a competitive fashion. At a half-maximal concentration of cGMP, the  $K_i$  values for  $R_p$ -8-pCPT-cGMPS and  $R_p$ -8-Br-PET-cGMPS determined from Dixon plots were 0.15 and 0.45  $\mu\text{M}$ , respectively (Fig. 5).

To allow a direct comparison between the kinetic properties of cGK II and cGK I, we determined the analog specificity of purified bovine lung cGK I under the same conditions as used for cGK II. As shown in Fig. 6, pCPT-cGMP was a more potent stimulator of cGK II compared with cGK I, whereas the 8-Br-PET and PET derivatives of cGMP were relatively selective activators of cGK I. Similarly,  $R_p$ -Br-PET-cGMPS was found to preferentially inhibit cGK I, whereas  $R_p$ -pCPT-cGMPS displayed an opposite selectivity. Furthermore, the  $R_p$ -cGMPS analogs failed to completely inhibit the cGMP-stimulated activity of cGK I (maximal inhibition 70%, data not shown) due to

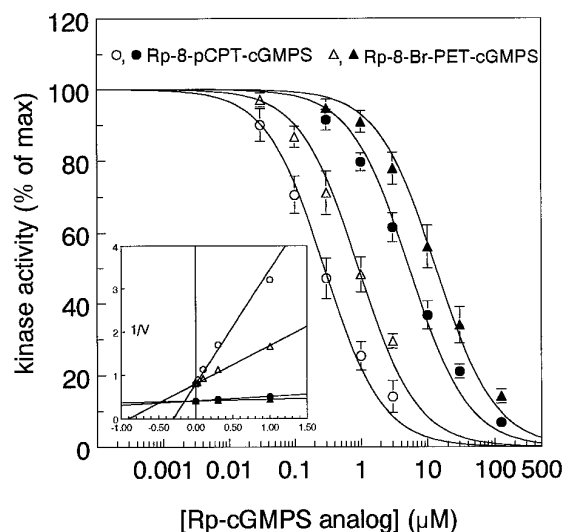


FIG. 5. Inhibition of cGMP-stimulated protein kinase activity in rat intestinal brush border caps by  $R_p$  diastereoisomers of phosphorothio-cGMP analogs. Phosphorylation of 2A3 by rat intestinal brush border caps was measured using 300  $\mu\text{M}$  ATP in the presence of either 0.3  $\mu\text{M}$  (open symbols) or 3  $\mu\text{M}$  (closed symbols) cGMP plus various concentrations of  $R_p$ -8-pCPT-cGMPS ( $\circ$ ,  $\bullet$ ) or  $R_p$ -8-Br-PET-cGMPS ( $\Delta$ ,  $\blacktriangle$ ). Data are expressed as percentage of the phosphotransferase activity in the absence of  $R_p$ -cGMPS analogs and are the means  $\pm$  S.E. of three experiments. Inset, Dixon plot for the determination of  $K_i$  values (abscissa, concentration of  $R_p$ -cGMPS in  $\mu\text{M}$ ; ordinate, reciprocal value of enzyme activity expressed in  $\text{nmol}^{-1}\cdot\text{min}\cdot\text{mg}$  protein).

agonistic effects of these partial antagonists occurring at higher concentrations. A similar agonistic effect of  $R_p$ -pCPT-cGMPS concentrations in the millimolar range has been reported previously for both cGK I and II (17).

**Effects of cGMP Analogs on  $\text{Cl}^-$  Secretion in Intestinal Epithelium**—To evaluate whether the analog specificity of cGK II determined *in vitro* has any relevance for its activation of physiological substrates in intact epithelial cells, we examined the effects of membrane-permeant cGMP analogs on intestinal  $\text{Cl}^-$  secretion as monitored by short-circuit current ( $I_{sc}$ ) measurements in rat cecum in Ussing chambers. In this intestinal segment cGK II was found to be colocalized with the cystic

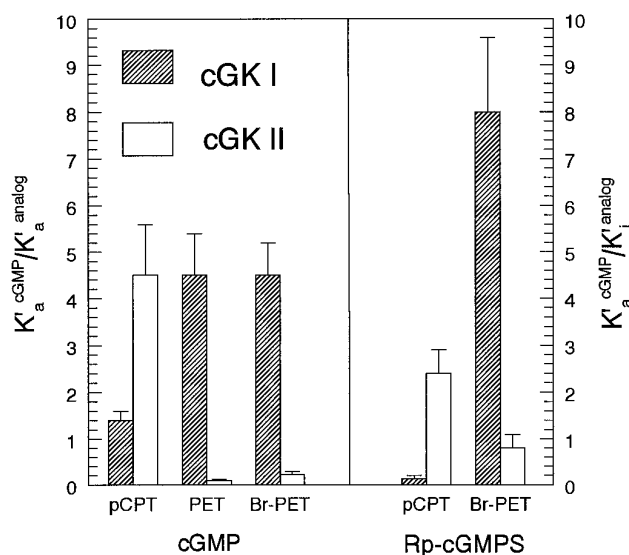


FIG. 6. Comparison of the specificity of cGK I and cGK II for cGMP analogs. Apparent  $K_a$  values were determined from dose-response curves of 2A3 phosphorylation by cGK II in rat intestinal brush border caps (open bars) and purified bovine lung cGK I (hatched bars) in the presence of 300  $\mu$ M ATP and various concentrations of cGMP or the cGMP analogs indicated (cf. Fig. 3 and Table II). Apparent  $K_i$  values for the  $R_p$  analogs of cGMPs were determined at a half-maximally stimulating concentration of cGMP (0.3  $\mu$ M) from Dixon plots (cf. Fig. 5), after correction for a basal stimulatory effect of  $R_p$  analogs in the absence of cGMP in case of cGK I. Data are expressed relative to the apparent  $K_a$  for cGMP in each experiment and are means  $\pm$  S.E. of three experiments.

fibrosis trans-membrane conductance regulator  $Cl^-$  channel in the apical membrane of the crypt cells, in line with the relatively large increase in  $Cl^-$  secretion provoked by the cGK-specific agonist 8-Br-cGMP in this tissue (12). As expected from the relatively low apparent  $K_a$  of cGK II for 8-pCPT-cGMP, micromolar concentrations of this analog could stimulate  $I_{SC}$  (Fig. 7 and Table II), whereas 8-Br-PET-cGMP and PET-cGMP, relatively poor agonists of cGK II *in vitro*, showed  $EC_{50}$  values in the submillimolar range (Table II). The difference between the cGMP analogs cannot be caused by a difference in membrane permeability since 8-Br-PET-cGMP and PET-cGMP have a higher or equal lipophilicity, respectively, compared with 8-pCPT-cGMP (Table II). Furthermore, if the different lipophilicities of the  $R_p$  cGMPs analogs were taken into account, their inhibitory effects on 8-pCPT-cGMP-stimulated  $I_{SC}$  (see Fig. 7) correlated well with their  $IC_{50}$  values found in *in vitro* cGK II protein kinase assays (Table II), further corroborating the notion that the analog specificity of cGK II in intact cells and in isolated membranes is very similar.

#### DISCUSSION

Type II and type I cGK isoenzymes are likely to play distinct physiological roles, as judged from their differences in tissue expression, subcellular localization, and functional properties *in vitro* (2–5, 12–18). These different characteristics could in principle be exploited to assess the involvement of a specific cGK isoform in a cGMP-regulated cellular process. However detailed analyses of the molecular properties of cGK II have so far been primarily confined to recombinant and/or purified enzyme preparations, and the results may not be representative for cGK II in its native membrane environment, e.g. due to possible differences in posttranslational modifications, loss of modulatory components, proteolysis or protein oxidation. Indeed pronounced differences in the membrane localization, oligomerization status, and kinetic properties of various preparations of cGK II have been reported (3–5, 16–18). These

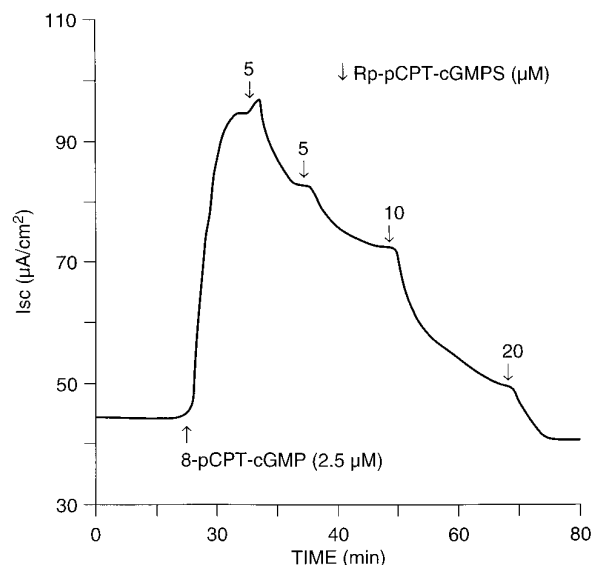


FIG. 7. Inhibition of 8-pCPT-cGMP-stimulated short-circuit current in rat cecum by  $R_p$ -8-pCPT-cGMPs. Short-circuit current ( $I_{SC}$ ) was measured in stripped rat cecum in an Ussing chamber. At the time points indicated with an arrow a single dose of 8-pCPT-cGMP (2.5  $\mu$ M) was added to the serosal site, followed by sequential serosal additions of  $R_p$ -8-pCPT-cGMPs ( $\mu$ M concentrations shown) resulting in final cumulative concentrations of 5, 10, 20, and 40  $\mu$ M.

apparent discrepancies urged us to re-evaluate some of the structural and functional properties of cGK II in its native environment, the apical membrane of intestinal epithelial cells.

Pig cGK II was characterized as a dimer in brush border membranes, as was soluble mouse cGK II expressed in Sf9 cells (16). The monomeric behavior of purified pig cGK II described previously (3) appeared to result from the presence of catalytically active C-terminal 75- and 70-kDa proteolytic fragments in this preparation, most likely generated during purification by a specific endogenous protease characterized previously in intestinal brush borders (26). The observation that these proteolytic forms are monomeric indicates that dimerization of cGK II is critically dependent on a structural domain located within the first 100 amino acids. This is consistent with a dimerization role for the leucine zipper motif recognized in this region of cGK II (4). The leucine-isoleucine repeat is conserved in all cGK sequences examined (4) and has been shown to dimerize an N-terminal fragment of cGK I $\alpha$  *in vitro* (27). Both the detergent-solubilized recombinant and endogenous cGK II observed in the present study were found to have significantly larger Stokes radii and the recombinant cGK II a smaller sedimentation coefficient as compared with either cGK I (Table I; 3, 16, 28) or the soluble recombinant mouse cGK II described by Gamm *et al.* (16). This suggests that the molecular shape of solubilized particulate cGK II is more elongated than that of cGK I and very different from the almost spherical form of soluble mouse cGK II. However, the primary amino acid sequences of rat intestinal and mouse brain cGK II (4, 5, correction in Ref. 16) are nearly identical, suggesting that the higher degree structures of these proteins should be quite similar. Perhaps their shapes may be influenced by different N-terminal modifications, since rat intestinal cGK II appears to be myristoylated (18), whereas recombinant mouse brain cGK II was modified with an N-terminal histidine tag (16). Certainly these differences may be one explanation for the membrane localization of endogenous pig and rat intestinal cGK II (3, 12), as well as rat intestinal cGK II expressed in HEK 293, Sf9, and COS cells (4, 17, 18), whereas mouse brain cGK II expressed in COS and Sf9 cells remained soluble (5, 16).

Another discrepancy between the various preparations of cGK II is their sensitivity to cGMP and cGMP analogs. Part of these differences might be explained by variations in assay conditions, in particular the ATP concentration, which could shift the apparent  $K_a$  of cGK II for cGMP by as much as 10-fold (Fig. 4). A similar modulatory effect of ATP was observed for cGK I (Fig. 4B), in line with previous reports describing downward shifts in binding affinity of cGK I and cAMP-dependent protein kinase type I for cGMP and cAMP, at concentrations of ATP around the  $K_m$  (29–31). This suggests a similar mode of interaction between the ATP-bound catalytic domain and the regulatory domain of all three cyclic nucleotide-dependent protein kinases, resulting in an apparent competition between ATP and cyclic nucleotides (31). However, the effect of ATP on cGMP affinity in kinase activity measurements was particularly prominent in case of cGK II which displayed an unusually high  $K_m$  for ATP (400  $\mu\text{M}$ ) in comparison to cAMP-dependent protein kinase (5  $\mu\text{M}$ ; Ref. 31) and cGK I (66  $\mu\text{M}$ , this study; 20–50  $\mu\text{M}$ ; Refs. 29 and 30).

After correction for the different ATP concentrations employed in each assay (using the data plotted in Fig. 4B), the apparent  $K_a$  for cGMP of endogenous rat cGK II was found to be approximately 3-fold higher than that of recombinant rat cGK II purified from Sf9 cells (17), however, 3-fold lower than that of either recombinant rat cGK II solubilized from COS-1 cells (18) or histidine-tagged recombinant mouse cGK II purified from Sf9 cells (16), and similar to the apparent  $K_a$  for recombinant mouse cGK II expressed in COS cells (5). The lack of consistent or major differences between native cGK II and the various preparations of cGK II obtained after solubilization or purification, suggests that the characteristics of cGK II are not dramatically influenced by the membrane environment. Indeed when tested directly, solubilization of rat cGK II expressed in 3T3 fibroblasts resulted only in a slight (1.6-fold) increase in its apparent  $K_a$  for cGMP.

A reasonable but not perfect correspondence was also found between the effects of cGMP analogs on endogenous cGK II and the purified recombinant enzymes described previously. For instance PET-cGMP had a low affinity for both endogenous rat cGK II (4.2  $\mu\text{M}$ ; Table II) and for recombinant mouse cGK II purified from Sf9 cells (4.7  $\mu\text{M}$ ; Ref. 16). In contrast, PET-cGMP had the same high affinity (0.06  $\mu\text{M}$ ) as had cGMP (0.04  $\mu\text{M}$ ) for the recombinant rat enzyme purified from Sf9 cells (17). However, the latter preparation, similar to endogenous rat cGK II, was stimulated by relatively low levels of 8-pCPT-cGMP (3.5 and 80 nM, respectively; Ref. 17). Furthermore, the endogenous rat enzyme, similar to recombinant rat and mouse cGK II purified from Sf9 cells, was inhibitable by  $R_p$  analogs of cGMPs.

The inhibition of exogenous substrate phosphorylation by  $R_p$  analogs clearly differs from their stimulatory effects on cGK II autophosphorylation in brush border membranes (9). The apparent  $K_a$  for cGMP was also different for substrate- and autophosphorylation, the latter being 5–10-fold lower. Since the apparent  $K_a$  of cGMP for autophosphorylation was close to the  $K_d$  of cGMP determined previously for the high affinity site of cGK II (3), it is tempting to suggest that autophosphorylation is stimulated by occupancy of only one of the cyclic nucleotide binding sites available (*i.e.* the “high affinity” or “slow” site), whereas substrate phosphorylation apparently requires both sites for optimal activity. Whether autophosphorylation has any effect on the kinetic properties of cGK II as described for cGK I $\alpha$  and cGK I $\beta$  (32, 33) remains to be investigated. However, a substantial influence of autophosphorylation on the kinetic parameters determined in this study is unlikely considering the poor stoichiometry of cGK II autophosphorylation

within the relatively short incubation periods used (3) and the observed linearity of the kinase activity with time, *i.e.* the absence of a lag time (see “Experimental Procedures”).

The correlation observed here between the effects of cGMP analogs on substrate phosphorylation and on intestinal  $\text{Cl}^-$  secretion indicates that the characteristics of cGK II determined *in vitro* are relevant for its functioning under physiological conditions. This implies that the cGMP analog specificity of cGK II, which is clearly distinct from that of cGK I (according to this and previous studies; see refs. 14, 16, 17, 34, and 35), can fortuitously be used to discriminate between the functional effects of each isotype both in *in vitro* assays and in intact cells. A potency order 8-pCPT-cGMP  $\gg$  8-Br-PET-cGMP > PET-cGMP would be diagnostic for cGK II, whereas the order 8-Br-PET-cGMP  $\geq$  PET-cGMP > 8-pCPT-cGMP would indicate a role for cGK I ( $\alpha$  or  $\beta$ ).

Finally our observation that  $R_p$ -cGMPs analogs, with some degree of specificity, can inhibit cGK II-controlled functions in intact tissues, as well as cGK I action described earlier (36, 37), indicates that these compounds are more generally applicable as tools to demonstrate the involvement of cGK I or II in specific physiological processes. For example, in a separate study of the mechanism of action of heat-stable enterotoxin (STa) in rat intestinal epithelium,  $R_p$ -8-pCPT-cGMPs appeared also able to inhibit the STa-induced  $\text{Cl}^-$  secretion (15). These results, together with recent cGK II knockout mouse data (7), strengthen the concept that in native intestine, cGK II plays an important role in the salt and water secretion initiated by activation of guanylyl cyclase C by STa, or guanylin, the putative endogenous ligand of GC-C (9–11).

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