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### Research Article

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# Role of cGMP-Kinase II in the Control of Renin Secretion and Renin Expression

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## Abstract

To investigate the roles of the cGMP-dependent protein kinases (cGKs) in the control of the renin system, we studied the regulation of renin in cGKI- or cGKII-deficient mice in vivo and in vitro. Renal renin mRNA levels both under stimulatory (low-salt diet plus ramipril) and inhibitory (high-salt diet) conditions were not different between wild-type and cGKI<sup>-/-</sup> mice, but were significantly elevated in cGKII<sup>-/-</sup> mice under all experimental conditions. In primary cultures of renal juxtaglomerular cells (JG) established from wild-type, cGKI<sup>-/-</sup>, and cGKII<sup>-/-</sup> mice, the adenylate cyclase activator forskolin stimulated renin secretion similarly in all genotypes tested. 8-bromo-cGMP attenuated basal and forskolin-stimulated renin secretion in cultures from wild-type and cGKI<sup>-/-</sup>, but had no effect in cells isolated from cGKII<sup>-/-</sup> mice. Activation of cGKs by 8-bromo-cGMP decreased renin secretion from the isolated perfused rat kidney, independent of prestimulation by  $\beta$ -adrenoreceptor activation, macula densa inhibition, reduced perfusion pressure, or by a nominally calcium-free perfusate. Taken together, these findings suggest that activation of cGKII has a general inhibitory effect on renin secretion from renal JG cells. (J. Clin. Invest. 1998; 102:1576–1582.)

**Key words:** renin mRNA • juxtaglomerular cell • isolated perfused kidney • cGKII • knockout

## Introduction

The synthesis and the secretion of renin from renal juxtaglomerular (JG)<sup>1</sup> cells, as the main site of renin expression, is controlled by several second messenger pathways. The pathway best established to date is cAMP, which stimulates both the exocytosis of renin as well as renin mRNA levels (1–3). The natural opponent to cAMP in the cellular control of the renin system appears to be a calcium-related pathway that could involve protein kinase C activity (1–3). There is also evidence that cGMP may participate in the control of the renin system (2, 4, 5). The precise role of cGMP in this context, however, is

highly controversial. A review of the effects of factors elevating cGMP levels in JG cells, such as endothelium-derived nitric oxide (NO) or atrial natriuretic peptide, reveals a scatter of contradictory results. Thus, NO has been designated both a stimulator (6–15) and an inhibitor of renin secretion (16–21) in vivo and in vitro. Similar contradictory data—stimulation (22–24) and inhibition (25–28) of renin secretion—have been obtained for atrial natriuretic peptide. The data from studies on the effects of stable, membrane-permeable cGMP analogues is more consistent and generally reflect inhibition of renin secretion from isolated perfused kidneys, kidney slices, or isolated JG cells (18, 27, 29–32). Inhibition of renin secretion by cGMP could, in principle, be mediated by three different cGMP receptors: the cGMP-dependent protein kinases (cGKs), cGMP-gated ion channels, or cGMP-regulated phosphodiesterases (33–36). Because commonly used, membrane-permeable, stable cGMP analogues have a high affinity for cGKs, but only a low affinity to cAMP-phosphodiesterases (37), and because so far no evidence exists for cGMP-gated ion channels in JG cells (38), it appears reasonable to assume that the inhibitory effect of cGMP analogues on renin secretion is mediated by cGKs. JG cells contain both cGK subtypes, named cGKI (39, 40) and cGKII (41, 42) with strikingly different intracellular distributions. Whereas cGKI is localized in the cytosol, cGKII occurs predominantly in association with renin storage granules (43).

In view of the foregoing, the aim of the present study was to examine the roles of the two cGK subtypes in the control of renin secretion by studying the regulation of the renin system in tissues of cGKI<sup>-/-</sup> and cGKII<sup>-/-</sup> mice (44, 45), and to establish which of the different pathways regulating renin secretion at the organ level involves negative control by cGKs. To this end, we also studied in more detail the effect of cGK activation on renin secretion stimulated by classic conditions using the isolated perfused rat kidney model.

Our results suggest that cGKII exerts a general negative control function on the renin system both in vivo and in vitro.

## Methods

**Animals.** The experiments were conducted in 10–16-wk-old cGKI<sup>-/-</sup> (44) and cGKII<sup>-/-</sup> (45) mice. Litter-matched wild-type (+/+) mice were used as controls. Strain background (C57Bl/6  $\times$  129 Sv) of the <sup>-/-</sup> and <sup>+/+</sup> animals were identical. Eight mice of each genotype were assigned to three groups: (a) Control animals received normal chow (0.6% NaCl) and remained untreated; (b) Animals with high-salt diet. Mice were maintained for 10 d on chow balanced in all respects except for a high sodium content (4% NaCl, Altromin); and (c) Animals with low-salt diet and ramipril treatment. Mice were kept on low-salt diet (0.02% NaCl, Altromin) for 10 d. For the last 3 d, the converting enzyme inhibitor ramipril (10 mg kg<sup>-1</sup> day<sup>-1</sup>) was given in the drinking water.

At the end of the experiments the animals were killed by decapitation. The kidneys were removed rapidly and frozen in liquid nitrogen. The organs were stored at  $-80^{\circ}\text{C}$  until isolation of total RNA, which was extracted from the frozen kidneys as described by Chomczynski and Sacchi (46).

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1. Abbreviations used in this paper: cGK, cGMP-dependent kinase; JG, juxtaglomerular cell; NO, nitric oxide.

**Determination of renin mRNA by RNase protection assay.** Renin mRNA was measured by an RNase protection assay. For detection of preprorenin mRNA, an antisense RNA probe suitable for detecting mRNA levels from the renin-1 gene (ren-1), was generated by in vitro transcription of the plasmid vector pSP73 (Promega-Serva, Heidelberg, Germany), containing a PCR-derived fragment of mouse preprorenin cDNA (47). The 194-bp fragment, amplified by the upstream primer (5'-atg aag ggg gtc ttc tgt ggg gtc-3'; binding at 810–832 bp) and the downstream primer (5'-atg cgg gga ggg tgg gca cct g-3'; binding at 981–1,003 bp), was cloned in a BamHI/EcoRI-digested pSP73 vector using standard protocols. Linearization with HindIII and in vitro transcription with SP6 RNA polymerase yielded a 248-bp fragment. Hybridization, RNase digestion, phenol/chloroform extraction, and acrylamide electrophoresis are described in detail elsewhere (14).

**Determination of cytosolic  $\beta$ -actin by RNase protection assay.** The presence of cytosolic  $\beta$ -actin mRNA was measured by an RNase protection assay. The upstream primer (5'-cca act ggg aeg cat g-3'; binding at 152–168 bp) and downstream primer (5'-tgg cgt gag gga gag cat-3'; binding at 428–445 bp) were used to amplify a 293-bp fragment of mouse  $\beta$ -actin cDNA (48). Cloning in a BamHI/EcoRI-digested pSP73 plasmid, linearization with HindIII, and in vitro transcription with SP6 RNA polymerase yielded a 347-bp antisense RNA transcript.  $\beta$ -actin mRNA was used as a standard RNA for controlling the quality of the RNA preparation. Total RNA (1  $\mu$ g) was hybridized under the conditions described previously (14).

**Renin secretion from cultured JG cells.** Primary cultures of JG cells were established from wild-type, *cGKI*<sup>-/-</sup>, and *cGKII*<sup>-/-</sup> mice. The isolation of mouse JG cells has been described in detail elsewhere (32). Renin secretion rates were estimated from the appearance rate of renin in the culture medium. To minimize differences among different cell culture preparations, renin secretion rates were calculated as fractional release of total renin (i.e., renin activity released/[renin activity released + renin activity remaining in the cells]). The generated angiotensin I was determined by radioimmunoassay (Sorin, Biomedica, Düsseldorf, Germany).

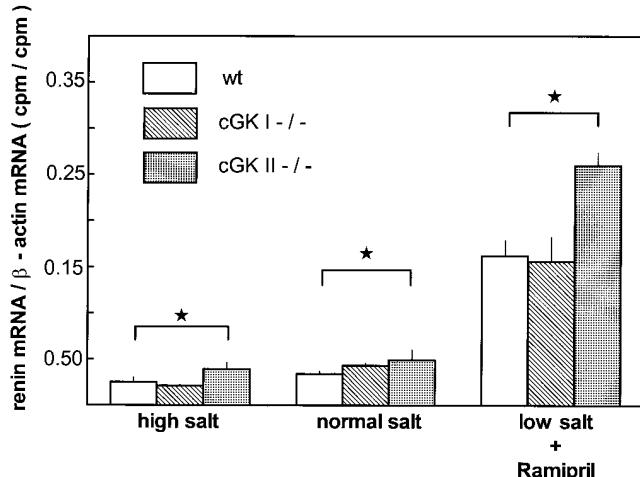
**Renin secretion from isolated perfused kidneys.** Male Sprague-Dawley rats (250–300 g body weight; Indianapolis, IN) having free access to commercial pellet chow and tap water were obtained from the local animal house and used throughout. Preparation and kidney perfusion were performed in a recycling system as described in detail previously (30). After establishing the reperfusion loop, perfusate flow rates usually stabilized within 15 min. Samples for the determination of renin activity were taken at 5-min intervals. Renin secretion rates were calculated from the arterio-venous differences of renin activity and the perfusate flow rate. The perfusate samples were diluted 1:5 in buffer and were incubated for 1.5 h at 37°C with plasma from bilaterally nephrectomized male rats as renin substrate. The generated angiotensin I was determined by radioimmunoassay (Sorin, Biomedica). Five kidneys were used for each experimental protocol.

**Statistics.** Significance levels between kidneys were calculated with ANOVA and Bonferroni's correction. Student's paired *t* test was used to calculate levels of significance within individual cell preparations. Student's paired *t* test was used also to calculate levels of significance within individual kidneys. A *P* value of 0.05 was considered significant.

## Results

**In vivo experiments.** To study the possible roles of cGKI and cGKII in the control of the renal renin system in vivo, mice lacking either cGKI or cGKII and the corresponding wild-type controls were subjected to maneuvers either stimulating or inhibiting renal ren-1 mRNA levels determined by specific RNase protection.

Under a standard salt diet (0.6% NaCl), ren-1 mRNA levels were not different between wild-type and *cGKI*<sup>-/-</sup> mice,



**Figure 1.** Renin mRNA expression in the kidneys of wild-type mice and in mice lacking the cGMP-dependent kinase, types I or II (*cGKI*<sup>-/-</sup> and *cGKII*<sup>-/-</sup>) kept on a normal salt diet, a high-salt diet, or on a low-salt diet plus ramipril. Renin-mRNA values were standardized to  $\beta$ -actin mRNA, which was not different between the different mouse genotypes, nor between the different treatment groups. Data are means  $\pm$  SE of eight mice in each group. \**P* < 0.05.

whereas in *cGKII*<sup>-/-</sup> mice, ren-1 mRNA was increased by  $\sim$  50% (Fig. 1). A high-salt diet (4%) for 10 d decreased renin mRNA significantly to  $\sim$  50% of normal in both wild-type and in *cGKI*<sup>-/-</sup> mice. Although in the *cGKII*<sup>-/-</sup> mice, renin mRNA levels were also lowered by a high-salt intake, renin mRNA concentrations were still 60% higher than in wild-type and *cGKI*<sup>-/-</sup> mice (Fig. 1). Feeding a low-salt diet (0.02%) for 10 d combined with the ACE inhibitor, ramipril (10 mg  $\text{kg}^{-1}$  day $^{-1}$ ), for the last 3 d increased renin mRNA levels significantly by about fivefold compared with normal in both wild-type and *cGKI*<sup>-/-</sup> mice, whereas in *cGKII*<sup>-/-</sup> mice, renin mRNA levels were only  $\sim$  40% higher than in wild-type and *cGKI*<sup>-/-</sup> mice (Fig. 1). Taken together, the regulation of renal renin mRNA was normal in *cGKI*<sup>-/-</sup> mice. In *cGKII*<sup>-/-</sup> mice, the regulation of renin mRNA by salt intake was also quite normal, although renin gene expression appeared to be enhanced in proportion under each of the conditions examined.

**Experiments with cultured juxtaglomerular cells.** To study the effects of the lack cGK on renin release from the JG apparatus, we prepared primary cultures of JG cells from wild-type, *cGKI*<sup>-/-</sup>, and *cGKII*<sup>-/-</sup> kidneys. The basal renin release rates of JG cells isolated from wild-type and from *cGKI*<sup>-/-</sup> mice were similar, whereas that from *cGKII*<sup>-/-</sup> tended to be somewhat higher (Fig. 2). Renin secretion was stimulated similarly in JG cell cultures of all three mouse genotypes by the adenylate cyclase activator forskolin (Fig. 2). Conversely, activation of cGK by 8-bromo-cGMP (100  $\mu$ M) attenuated basal and forskolin-induced renin secretion significantly in JG cell from wild-type and *cGKI*<sup>-/-</sup> mice, but was without effect in cultures derived from *cGKII*<sup>-/-</sup> mice (Fig. 2). Because the inhibitory effect of 8-bromo-cGMP on renin secretion in wild-type and *cGKI*<sup>-/-</sup> cultures could be reversed by higher concentrations of forskolin (Fig. 2), it was of interest to compare the dependency of the inhibitory effect of 8-bromo-cGMP on

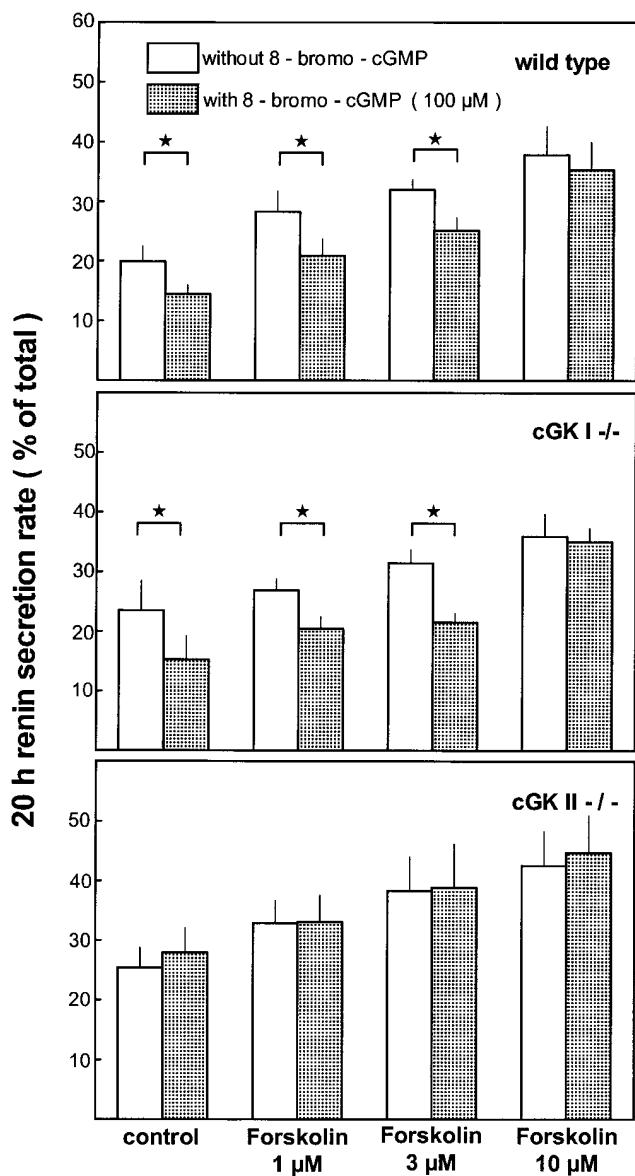


Figure 2. Renin secretion rates from primary cultures of renal JG cells prepared from wild-type, *cGK I* *-/-*, and *cGK II* *-/-* mice. Renin secretion rates were determined over a 20-h incubation period with vehicle, increasing doses of forskolin (1, 3, and 10  $\mu$ M) and 8-bromo-cGMP (100  $\mu$ M). Renin secretion rates are given as fractional release of total renin activity stored in the cells. Data are means  $\pm$  SE of five experiments, each done with different cell preparations. Each experiment represents the mean of quadruplicate culture wells. \* $P < 0.05$ . Total renin activity of the cultures was 29  $\pm$  6, 32  $\pm$  8, and 38  $\pm$  6 mg ANGI  $\times$  h $^{-1}$   $\times$  mg protein $^{-1}$  for cells isolated from wild-type, *cGK I* *-/-*, and *cGK II* *-/-* mice, respectively.

the concentration of forskolin between wild-type, *cGK I* *-/-*, and *cGK II* *-/-* cultures. As shown in Fig. 2, an inhibitory effect of 8-bromo-cGMP in *cGK II* *-/-* cultures was absent in the whole concentration range of forskolin used in this study.

Because the foregoing observations strongly imply an inhibitory effect of cGKII on the renin system, it appeared of further interest to investigate which of the multiple pathways involved in the control of renin secretion on the organ levels

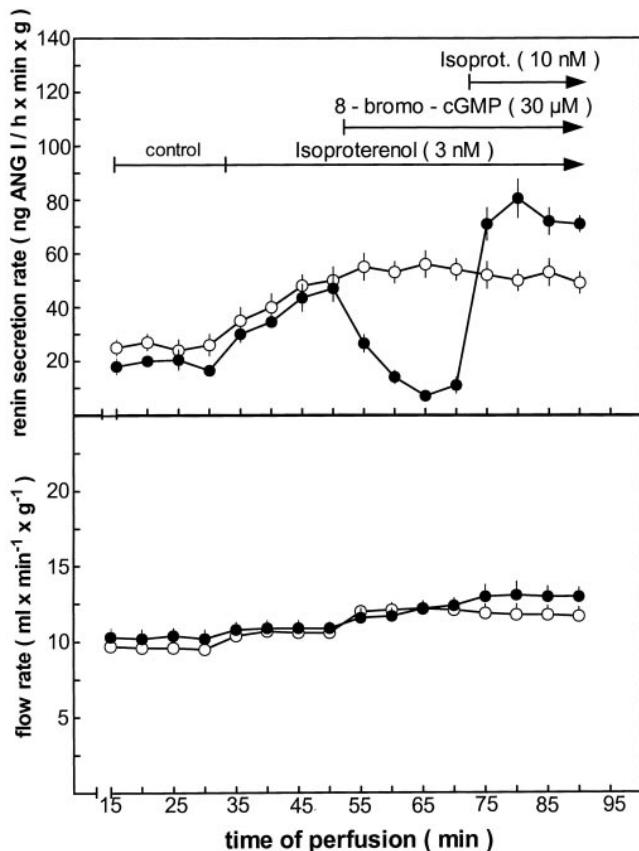


Figure 3. Effects of isoproterenol (3 and 10 nM) and of 8-bromo-cyclic GMP (30  $\mu$ M) on renin secretion and renal flow rate from isolated rat kidneys perfused at 100 mmHg (closed circle). Samples were taken at 5-min intervals during the different experimental periods, the duration of which is indicated in the upper part of the figure. Time control without 8-bromo-cGMP and isoproterenol (10 nM) perfusion (open circle). For determination of significant differences, renin secretion rates obtained within a certain experimental period of each kidney were taken together and averaged. The experimental protocols were run with five different kidneys. Data are means  $\pm$  SE.

involves cGKII. This question was addressed in experiments in the isolated perfused rat kidney.

*Experiments with isolated perfused kidneys.* As shown in Fig. 3, the  $\beta$ -adrenoreceptor activator isoproterenol (3 nM) led to a sustained, threefold stimulation of renin secretion ( $P < 0.05$ ). In the presence of 8-bromo-cGMP (30  $\mu$ M), renin secretion rates significantly decreased ( $P < 0.05$ ) and returned to near basal values. Increasing the concentration of isoproterenol from 3 to 10 nM in the presence of 8-bromo-cGMP again increased renin secretion rates ( $P < 0.05$ ) (Fig. 3). The renal blood flow in this experiment showed a slight but significant increase when 8-bromo cGMP was added to the perfusate (Fig. 3, bottom).

Renin secretion is also controlled by an inhibitory signal from the macula densa cells, which generate this signal in proportion to their salt transport rate (49, 50). Inhibition of salt transport at the macula densa by the loop diuretic bumetanide (100  $\mu$ M) doubled renin secretion ( $P < 0.05$ ) (Fig. 4). In the presence of 8-bromo-cGMP, renin secretion rates decreased ( $P < 0.05$ ) and returned to slightly subnormal values, and in-

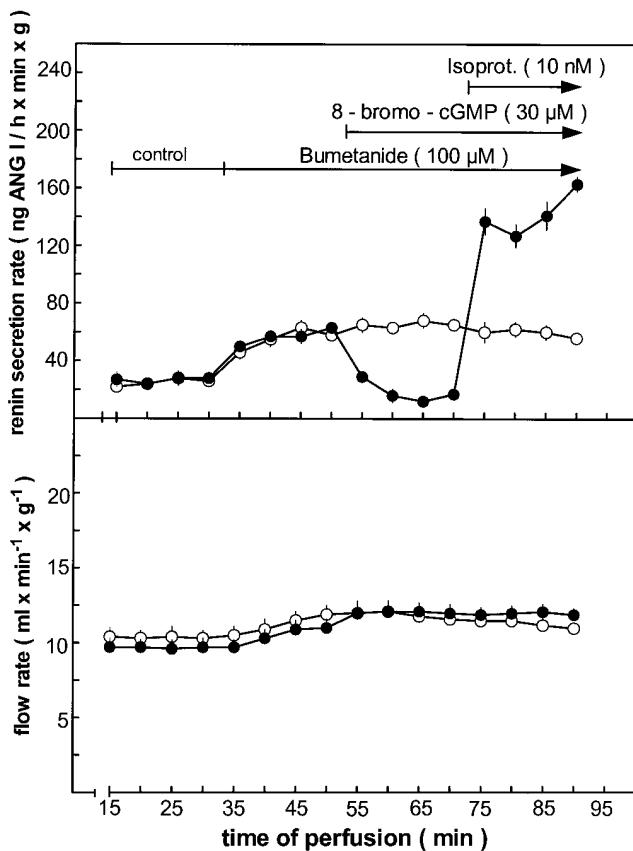


Figure 4. Effect of bumetanide (100  $\mu$ M), 8-bromo-cGMP (30  $\mu$ M), and of isoproterenol (10 nM) on renin secretion and renal flow rate from isolated rat kidneys perfused at 100 mmHg (closed circle). Samples were taken at 5-min intervals during the different experimental periods, the duration of which is indicated in the upper part of the figure. Time control without 8-bromo-cGMP and isoproterenol (10 nM) perfusion (open circle). For determination of significant differences, renin secretion rates obtained within a certain experimental period of each kidney were taken together and averaged. The experimental protocols were run with five different kidneys. Data are means  $\pm$  SE.

creased ( $P < 0.05$ ) again when isoproterenol (10 nM) was added to the perfusate in the presence of bumetanide and 8-bromo-cGMP (Fig. 4). Again, renal blood flow increased slightly ( $P < 0.05$ ) in the presence of 8-bromo-cGMP (Fig. 4, bottom).

Renin secretion is also under the control of the renal perfusion pressure, such that a fall of the perfusion pressure enhances renin secretion (1). As shown in Fig. 5, a reduction of the perfusion pressure from the standard pressure of 100 mmHg to 40 mmHg led to a significant decrease of renal flow rate and a 3.5-fold increase ( $P < 0.05$ ) of renin secretion. 8-bromo-cGMP (30  $\mu$ M) attenuated ( $P < 0.05$ ) this stimulation by low pressure to an about twofold stimulation (versus basal) of renin secretion (Fig. 5). Isoproterenol (10 nM) again increased ( $P < 0.05$ ) renin secretion to  $\sim 10$  times above basal values in the presence of 8-bromo-cGMP at a perfusion pressure of 40 mmHg (Fig. 5).

Calcium is thought to exert an inhibitory effect on renin secretion, and lowering the extracellular calcium concentration increases renin secretion in a variety of experimental prepara-

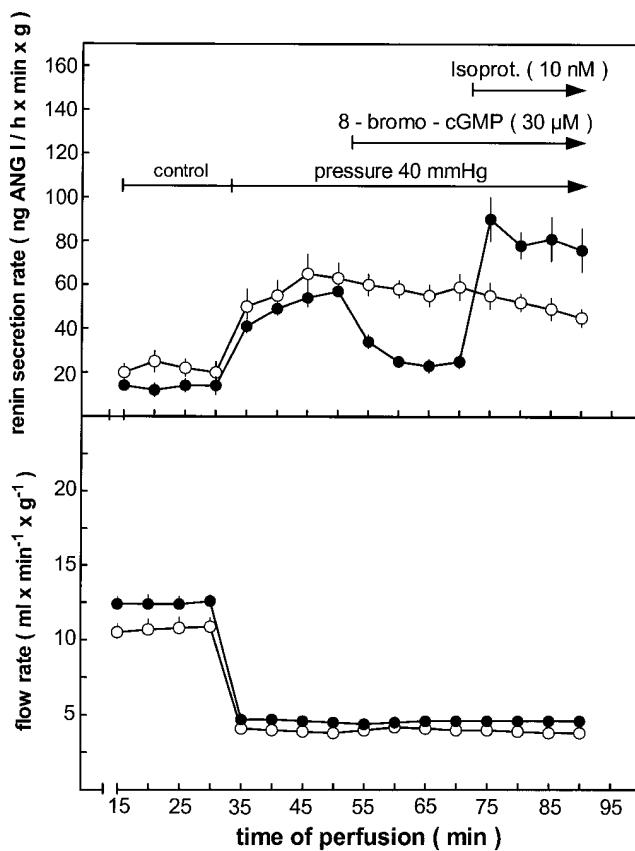


Figure 5. Effect of 8-bromo-cGMP (30  $\mu$ M) or isoproterenol (10 nM) on renin secretion and renal flow rate in isolated rat kidneys perfused at 40 mmHg (closed circle). Samples were taken at 5-min intervals during the different experimental periods, the duration of which is indicated in the upper part of the figure. Time control without 8-bromo-cGMP and isoproterenol (10 nM) perfusion (open circle). For determination of significant differences, renin secretion rates obtained within a certain experimental period of each kidney were taken together and averaged. The experimental protocols were run with five different kidneys. Data are means  $\pm$  SE.

tions (1). In our experiments, we lowered the extracellular calcium by using a nominally calcium-free perfusate supplemented with 0.5 mM EGTA, a calcium chelator. As shown in Fig. 6, lowering the extracellular calcium concentration led to a fourfold stimulation ( $P < 0.05$ ) of renin secretion (Fig. 6) and simultaneously to an increased blood flow. 8-bromo-cGMP again reversed the renin stimulation ( $P < 0.05$ ), and further addition of isoproterenol to the low-calcium, 8-bromo-cGMP-containing perfusate reestablished the stimulation ( $P < 0.05$ ) (Fig. 6).

## Discussion

Against a background of indirect evidence that cGK activity may exert an inhibitory control on the renin system, the aim of this study was to establish and characterize the specific role of the different cGK subtypes. Our findings now show that the regulation of the renal renin system, as reflected by renal renin mRNA levels, is completely unobtrusive in mice lacking cGKI, suggesting that cGKI either plays no major role in the control

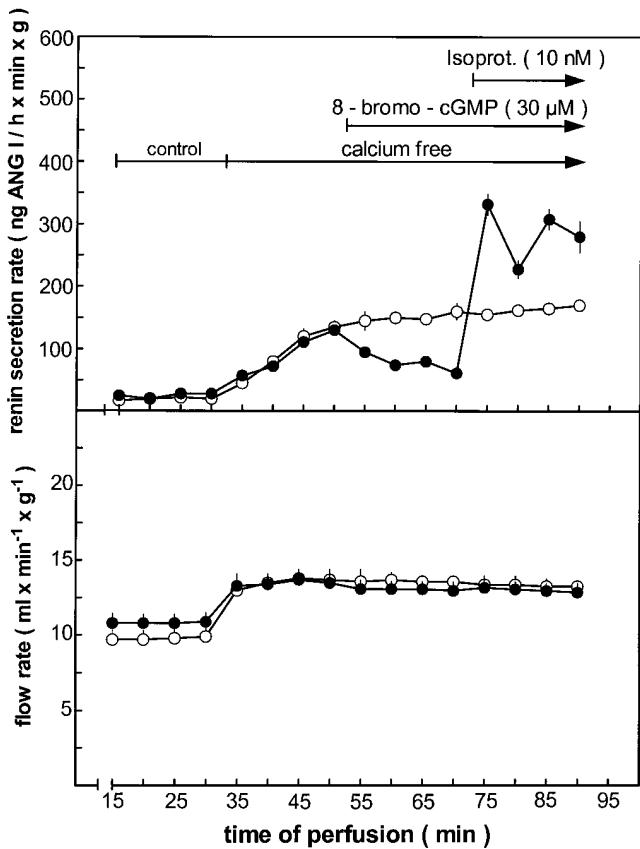


Figure 6. Effect of calcium-free perfusate, 8-bromo-cGMP (30  $\mu$ M) and isoproterenol (10 nM) on renin secretion and renal flow rate from isolated rat kidneys perfused at 100 mmHg (closed circle). Samples were taken at 5-min intervals during the different experimental periods, the duration of which is indicated in the upper part of the figure. Time control without 8-bromo-cGMP and isoproterenol (10 nM) perfusion (open circle). For determination of significant differences, renin secretion rates obtained within a certain experimental period of each kidney were taken together and averaged. The experimental protocols were run with five different kidneys. Data are means  $\pm$  SE.

of the renin system or that a potential effect induced by the lack of cGKI is completely compensated by other mechanisms. Because the regulation of renin secretion in isolated JG cells taken from *cGKI*<sup>-/-</sup> mice was also quite normal, it appears more reasonable to assume that cGKI plays, if at all, only a minor role in the renin system. In contrast, the inhibitory effect of cGK stimulation on renin secretion was completely absent in JG cells isolated from *cGKII*<sup>-/-</sup> mice. *In vivo* the gain of renin expression was significantly increased in *cGKII*<sup>-/-</sup> mice, whereas the principal regulation by salt or angiotensin II was maintained. These findings are compatible with the idea that cGKII exerts a more general inhibitory effect on the renin system and which is also active under *in vivo* conditions. Our findings, moreover, suggest that the inhibitory effect of cGKII activation is not irreversible but may be overcome by strong adenylate cyclase activation. cGKs exhibit a different cellular distribution in JG cells. Whereas cGKI is found in the cytosol, cGKII is associated with renin storage granules (43). This unequal distribution of cGKs within JG cells may explain why only cGKII plays an active role in the inhibition of the renin system (see below).

To obtain further information on the relevance of cGKII-mediated inhibition of renin secretion, we studied the effect of cGK activation on renin secretion prestimulated by different physiological maneuvers. cGK activation rather unselectively attenuated renin secretion stimulated by  $\beta$ -adrenoreceptor activation, macula densa inhibition, the renal baroreceptor mechanism, and reduction of the extracellular calcium concentration. In all instances, renin secretion could again be increased by strong adenylate cyclase activation by a higher concentration of isoproterenol, findings quite consistent with those made in isolated JG cells. Supportive evidence for our conclusions further exists from a study showing that G-kinase activator 8 p-CTP-cGMP inhibited renin secretion from microdissected renal afferent arterioles and the G-kinase inhibitor Rp-8 p-CTP-cGMP attenuated this inhibition (51).

Because cGK activation also inhibited renin secretion induced by low extracellular calcium, a condition for example under which the inhibitory effect of angiotensin II on renin secretion is completely abolished (52), it may be inferred that the inhibitory effect of cGKII is not mediated by the inhibitory calcium pathway controlling renin secretion and renin synthesis. Further studies will therefore be necessary to define the inhibitory effect of cGKII at the level of the JG cell. In view of the fact that cGKII, but not cGKI, colocalizes with renin storage granules (43), and that cGKII activation apparently exerts a general inhibitory effect on the exocytosis of renin, one may speculate that cGKII acts by inducing events in renin secretory granules that impair the exocytotic event. According to the chemiosmotic theory of renin secretion, swelling of renin storage vesicles induced by KCl influx is a crucial event for the initiation of exocytosis (53). Because the swelling of renin storage vesicle has been hypothesized to be controlled by protein kinase-mediated events, it is conceivable that cGKII is involved in this interplay of protein kinases. In accordance with such a concept are also previous findings that cGKs can influence the activity of protein phosphatases (54–58), and of potassium and/or chloride channels (54–59). Whatever the intracellular mode of action of cGKII in JG cells may be, the present data imply that activation of cGKII by cGMP in JG cells inhibits renin secretion. At first glance, this conclusion is not free from contradiction because, as mentioned above, native stimulators of cGMP formation in JG cells, such as NO or ANP, reportedly stimulate renin secretion. We have shown that a stimulatory cGMP effect on renin secretion is causally related to inhibition of cAMP degradation (60). Thus, cGMP appears to have a dual effect on renin secretion, namely, stimulation via a linkage to the cAMP pathway and inhibition via cGKII activation. This raises the question about the determinants of the stimulatory or the inhibitory cGMP pathway. The majority of investigations report that the overall effect of cGMP *in vivo* is stimulation, suggesting that the cAMP pathway normally predominates. Because the inhibitory effect of membrane permeable cGMP analogues on renin secretion from JG cells can be reversed by strong adenylate cyclase activity (Fig. 2), it appears as if the inhibitory pathway could be masked by high cAMP efficacy. Conversely, in experiments with isolated perfused kidneys, NO donors have been shown to exert an intensified inhibitory effect on renin secretion in presence of calcium-mobilizing factors such as angiotensin II (30), suggesting that states of enhanced cytosolic calcium and protein kinase C activity impair the renin secretion via cAMP pathway. Taken together, it appears as if the momentary efficacy of cAMP or

calcium and protein kinase C systems determines whether the stimulatory or the inhibitory pathway predominates, thus, controlling the overall effect of cGMP on the renin system.

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