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Increases of Guanosine 3′,5′-Monophosphate-related Enzymes in Kidneys of Developing Rats*

DETLEF SCHLONDORFF[30] AND WALTER TRIZNA

Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA

Summary

Guanylate cyclase, cGMP phosphodiesterase and protein kinase activities were determined in kidneys of developing and adult rats. Guanylate cyclase activities of crude kidney homogenates, 100,000 × *g* supernatant and pellet of 7- and 21-day-old and adult rats were determined (Table I). In the kidneys of 7-day-old rats activity was 162% of adult controls in the homogenates ($P < .001$), 144% in the soluble ($P < .005$) and 308% in the particulate fraction ($P < .001$). In 3-week-old rats activity was still significantly higher at 144% in the homogenate ($P < 0.02$) and at 225% in the particulate ($P < .001$). Phosphodiesterase activity for cGMP was 7488 ± 831 pmol cGMP/mg protein · min in 1-week-old and 7674 ± 1120 in 3-week-old rats *vs.* 4042 ± 122 in the adults ($P < .025$) (Table II).

Chromatography on Sephadex G-200 showed two peaks of cGMP-stimulatable protein kinase in both the adult and newborn kidney and in addition a minor peak of cGMP-stimulatable kinase in the newborn kidney only (Fig. 2).

Speculation

These results provide further evidence for altered cGMP metabolism during kidney development. Whether cGMP is directly involved in organ growth remains open to speculation.

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INTRODUCTION

The role of cAMP as an intracellular mediator of hormone actions has been well established (22). The role of cGMP, however, remains open to speculation. It has recently been hypothesized that cGMP may be involved in the regulation of cell growth and proliferation (7). Though this hypothesis has been challenged (19), a number of animal studies (2,3,8,12,15,24) indicate altered activity of the cGMP system during organ growth. For example, an increase in particulate guanylate cyclase activity over that of the respective control tissues has been demonstrated in fetal and neonatal rat liver and hepatomas (12,2), in transplantable kidney tumors (3) and in regenerating livers of adult rats (12,8). We h. recently reported marked increases in cGMP levels associated with parallel changes in guanylate cyclase activity in rat kidneys undergoing compensatory renal hypertrophy (24). In addition, we observed higher cGMP levels in kidneys of very young rats (4-7 days) than in weanling (3 weeks) or adult rats (24). We therefore postulated that cGMP might be involved in compensatory renal hypertrophy and neonatal kidney growth (24). Furthermore, Kuo has presented evidence for changes in the cGMP dependent protein kinase activity of developing heart, lung and brain in the guinea pig (15,16). The present studies were therefore undertaken to further explore the role of the cGMP system during neonatal kidney development. The results provide further support for altered cGMP metabolism during organ development.

Materials

(^{3}H) labeled cAMP (38.4 Ci/m mole)

(^{3}H) labeled cGMP (9.92 Ci/m mole)

(^{32}P) labeled ATP (10 Ci/m mole)

were purchased from New England Nuclear Corp., Boston, Mass. Arginine rich histone Type VIII S, slightly lysine rich histone Type VII, mixed histone Type II, all from calf thymus, and snake venom (ophiophagus hannah) were obtained from Sigma Chemical Company, St. Louis, Mo. Reagents for competition radioimmunoassay of cGMP were p based from Collaborative Research Inc., Boston, Mass. All other reagents and chemicals were obtained from various suppliers and were of the best grade available.

Animals

Adult male Sprague Dawley rats (200-250g) and female rats with their litters were obtained from Taconic Farms, Germantown, New York. Adult rats were maintained on standard rat chow and water ad libitum,while the suckling littermates were kept with their mothers. Animals reported in each group were always from the same litter and the 21 day olds were siblings of the 7 day old rats.

Methods

Preparation of Guanylate Cyclase. Kidneys were removed from ether anesthetized rats and immediately placed in ice-cold 0.9% NaCl solution. While it is known that ether anesthesia has no effect on cGMP levels in the kidney (10) in preliminary experiments we had examined the effect of ether anesthesia or decapitation on guanylate cyclase and protein kinase and obtained comparable results with either method. All subsequent steps were carried out at 0-4°. The renal capsule was stripped. In the 7 day old rats the whole kidney was used whereas in the 21 day old and adult rats a crosssection of approximately 100 mg tissue, containing cortex and medulla, was obtained. The tissue was homogenized by hand, 10 strokes each, in 9 volumes of 0.3M Mannitol, 5 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA using ground-glass homogenizer. (Kontes Glass Co., Vineland, N.J.). Soluble and particulate guanylate cyclases were separated by centrifugation at 100,000 x g for 60 min in a Beckman L2-65B ultracentrifuge (Beckman Laboratories, Inc., Fullerton, Ca.). For determination of the particulate enzyme fraction the pellet was resuspended by gently rehomogenizing it in a volume of fresh homogenization solution equal to that of the original homogenate. Guanylate cyclase assays of the respective preparations were then carried out immediately.

Assay for Guanylate Cyclase. Guanylate cyclase activity was measured by the method of Kimura and Murad (11), as previously described from this laboratory (24). The assay reaction mixture contained 50 mM Tris-HCl buffer pH 7.6, 10 mM theophylline, 0.1 mM dithiothreitol, 15 mM creatine phosphate, 20 µg of creatine phosphokinase (120-135 units per mg), 1 mM GTP, 2.5 mM $MnCl_2$ in a final volume of 100 µl. The reaction was started by the addition of GTP and incubations carried out at 37° for 4 min. All reactions were carried out in duplicate. In order to eliminate interassay variations samples that were to be compared were always incubated in the same assay.

In preliminary experiments we established that the reaction was linear with time for 5 min at the following protein concentrations used per assay: 100-300 µg for the homogenate, 40-160 µg for the soluble and 50-100 µg for the particulate. Less than 5% of added ^{3}H cGMP was hydrolyzed under these conditions. Reactions were terminated by adding 0.9 ml of 50 mM sodium acetate buffer pH 4.0 and heating for 3 min at 90°C. cGMP generated was directly assayed in duplicate by radioimmunoassay of Steiner et al (26) as previously described from this laboratory (24). Samples incubated without enzyme and with heat-inactivated enzyme served as blanks and amounted to 10-25% of the measured cGMP.

Preparation of Phosphodiesterase. Renal tissue was obtained and homogenized as described for the guanylate cyclase preparation except that the buffer consisted of approximately 9 volumes of a 10 mM Tris HCl (pH 7.5) containing 0.25M sucrose and 1 mM EDTA. The homogenate was centrifuged for 10 min at 16,000 x g and the supernatant fluid was either used immediately or stored at -70° until assayed for cGMP phosphodiesterase activity. No loss of activity was observed by this method of storage up to one month.

Assay for Phosphodiesterase. cGMP phosphodiesterase was assayed by a slight modification of the method of Rosen (23) as previously described from this laboratory (24). The standard reaction mixture contained 5 µM or 125 µM ^{3}H cGMP (containing 30,000 CPM), 10 mM $MgCl_2$, 1 mM dithiothreitol and 5 mM 5'GMP in a final volume of 0.1 ml of 50 mM Tris HCl (pH 8.1).

All assays were carried out in duplicate; variation between duplicate were less than 5%. Each value was corrected for the blank and results expressed as means of the corrected duplicate determination.

Preparation of Protein Kinase. Adult male Sprague Dawley rats and littermates of either sex (10 days old) were used. Kidneys were removed under ether anesthesia and rinsed in ice cold 50 mM potassium - phosphate buffer (pH 7.0) with 50 mM mercaptoethanol. All subsequent steps were performed at 4°. Kidneys were then minced in approximately 5 volumes of buffer and homogenized with 5 strokes of a machine driven (200 RPM) glass teflon homogenizer. The homogenate was centrifuged for 20 minutes at 30,000 x g and the supernatant solution used for protein kinase determination.

In experiments in which separation of the cGMP dependent and cAMP dependent protein kinase activity of kidney extracts was attempted, the 30,000 x g supernatant solution from one gram of fresh kidney tissue was adjusted to pH 5.4 by slow addition of 0.2N acetic acid as described by Kuo (17). After centrifugation for 10 min at 2,000 RPM the precipitate was resuspended in 2 ml of 50 mM potassium - phosphate buffer (pH 7) with 50 mM mercaptoethanol and charged onto a Sephadex G-200 column (55x2.5cm) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The column had previously been equilibrated with the 50 mM potassium - phosphate buffer (pH 7.0) and elution was performed with the same buffer at a rate of 15 ml/hour, collecting 2.5 ml fractions.

Crude protein kinase modulator from rat lung was prepared through the steps of boiling and acid precipitation by the method of Walsh et al (30).

Assay for Protein Kinase. Protein kinase was determined by the method of DeLange et al (4) as modified by Ehrlichman et al (5). The final reaction volume was 0.2 ml containing 50 mM potassium - phosphate buffer (pH 7.0), 50 mM mercaptoethanol, 2.5 mM theophylline, 40 µg histone type VII, 20 mM $MgCl_2$, 0.5 mM [^{32}P] ATP (containing 1-2 x 10^6 CPM), 0.3 mg bovine serum albumin and, when used, 100 pmol of cAMP or cGMP. After 10 min preincubation at 30° in the presence of appropriate amounts of the kinase preparation the reaction was started by the addition of ATP and carried out at 30° in a shaking water bath (100 cycles/min). Reactions were stopped by the addition of 1 ml of 10% trichloracetic acid (TCA) and 0.1 ml of 0.1% bovine serum albumin. After centrifugation for 10 min at 2,000 RPM the supernatant was discarded and the pellet dissolved in 0.1 ml of 1N NaOH and immediately reprecipitated with 2ml of 5% TCA. The precipitate was recovered by filtration over glass-fiber-cellulose filters and washed with 20 ml of 5% TCA. After drying the filters were counted in 10 ml of triton-toluene in a liquid scintillation counter.

Samples incubated without enzyme and with heat inactivated enzyme served as blanks and never accounted for more than 0.02% of the total counts. Assays were carried out in triplicate, corrected for respective blanks and the results expressed as means of these corrected triplicates. Assays performed on fractions obtained from the Sephadex column were carried out in duplicate. Reactions proceeded linearly up to 4 min at protein concentrations of 0.02-0.2 mg/assay for the homogenate, and up to 10 min for the 30,000 x g supernatant and the pH 5.4 precipitate at protein concentrations of 0.02-0.1 mg/assay and 0.04-0.2 mg/assay, respectively.

Protein determinations were done by the method of Lowry (18) and by the modification of Geiger and Bessman (6) when sulfhydryl reagents were present. The statistical significance of the difference between groups was assessed by Student's t-test for unpaired data.

RESULTS

Guanylate Cyclase Activity. Renal guanylate cyclase activities for the crude homogenate, 100,000 x g supernatant solution and pellet of 7 and 21 day old and adult rats are given in Table I. Because of the variation between assays performed on different days, values for the respective adult control group are given separately for each group. In the 7 day old animals the enzyme activity was significantly higher at 162% of the adult controls in the crude homogenate ($P<0.001$), at 144% in the soluble ($P<0.005$) and at 308% in the particulate ($P<0.001$) enzyme fractions. Enzyme activity of 3 week old animals was still significantly higher at 144% of adult control in the crude homogenate ($P<0.02$) and at 225% in the particulate fraction ($P<0.001$), while the soluble enzyme activities were not significantly different.

As the recovery of enzyme activity for soluble and particulate preparations consistently was above 100% in both the newborn (136 ± 14%) and adult (128 ± 15%) rats, the respective particulate and soluble enzyme preparations from two adult and from two 21 day old rats were recombined and in each experiment the guanylate cyclase activity in the reconstituted homogenate exceeded that of the original homogenate, the mean increase being 23.8% ± 9.3 with no obvious difference between the adult and newborn animals. The reason for this increase in enzyme activity is not apparent from our studies.

Phosphodiesterase Activity for cGMP. Phosphodiesterase activity for cGMP in the 16,000 x g supernatant of renal homogenate was measured at 5 µM and 125 µM cGMP concentrations and results are shown in Table II. Activity was significantly higher in the kidneys of 7 and 21 day old rats, at both substrate concentrations used. No difference in activity was observed between the 7 and 21 day old rats. In order to see whether these differences could be explained by different substrate affinities, as measured by Km, or different enzyme activities as measured by Vmax, kinetic studies were performed at cGMP concentrations of 0.5 µM to 100 µM comparing the 21 day old and adult animals. A double reciprocal plot showed standard Michaelis Menten kinetics for both the newborn and adult animals (Fig. 1), with no major differences in calculated Km values for cGMP, but higher enzyme activity calculated as Vmax in the 21 day old rat kidneys.

Protein Kinase Activity. In preliminary experiments it was established that slightly lysine rich histone Type VII showed the highest degree of phosphorylation by the 30,000 x g supernatant of kidney homogenates from adult and neonatal rats.

Renal protein kinase activity was higher in homogenates and 30,000 x g supernatants of adult as compared to newborn rats. Cyclic AMP consistently stimulated kinase activity while cGMP at the same concentration did not. Addition of a crude protein kinase inhibitor (40-60 µg/assay) prepared by the method of Walsh et al (30) inhibited cAMP stimulation by 40% but did not change the absence of a response to cGMP. Protein kinase activity in the pH 5.4 precipitate was comparable for newborn and adult rats under basal conditions (159 ± 25 versus 153 ± 25 pmol Pi/mg prot x 1 min) and after stimulation with cAMP (224 ± 45 versus 213 ± 55) or cGMP (196 ± 36 versus 170 ± 34).

The pH 5.4 precipitate from adult and newborn kidneys was subjected to gel chromatography on Sephadex G-200. The eluted fractions were then assayed for protein kinase activity in the absence and presence of cAMP and cGMP. As shown in Fig. 2A and B two major peaks of cAMP stimulatable protein kinase activity (fractions #32-40 and #50-60) eluted in similar positions for both the adult and newborn kidney and, in addition, a minor peak of cGMP stimulatable kinase (fractions #45-48) could be demonstrated in the newborn kidney only (Fig. 2B). Similar results were obtained when the 30,000 x g supernatant was subjected to Sephadex G-200 chromatography and assayed for protein kinase activity. (Results not shown.)

DISCUSSION

Our findings of increases in guanylate cyclase (mainly particulate), in phosphodiesterase activity for cGMP and in cGMP stimulatable protein kinase in kidneys of newborn rats are evidence for altered cGMP metabolism during normal kidney growth and are in agreement with similar observations made in other growing organs (2,3,8,12,15,16,24). As the kidney consists of many different structures and cell types, the proportions of which undergo marked changes during postnatal development, results obtained with whole kidney preparation have to be interpreted with caution, however. In the adult kidney soluble guanylate cyclase activity accounted for about 70% and particulate for 30% of activity, which is in close agreement with the values reported by Kimura and Murad (11). Neer and Sukiennik (21) found 70-80% of guanylate cyclase activity in the soluble fraction of renal medulla in the adult rat and we (24) and Criss et al (3) have reported a similar distribution pattern for the renal cortex in the adult rat. Thus, in the adult rat at least, there appears to be no difference in distribution of soluble to particulate guanylate cyclase in the cortex versus the medulla. It therefore seems unlikely that changes in relative contribution of these structures could explain the marked increase in particulate guanylate cyclase activity observed in the kidneys of 7 day old rats. It also seems unlikely that the different distribution between soluble and particulate guanylate cyclase activity would be caused by enzyme transposition as there is evidence that the soluble and particulate guanylate cyclase are different enzymes (11). The fact that Kimura and Murad (12) reported increased particulate guanylate cyclase activity in fetal and neonatal rat liver makes it attractive to speculate that our findings are not explained by relative changes in composition of the kidney, but that they reflect a difference in cGMP metabolism during organ development.

Phosphodiesterase activity for cGMP was also elevated in the kidneys of newborn as compared to the adult rats. This was evident at substrate concentrations of 5 and 125 µM cGMP. Kinetic studies exhibited standard Michaelis Menten behavior and did thus not provide evidence for multiple cGMP phosphodiesterases in the 16,000 x g supernatant solution of kidney homogenates, a finding consistent with the previous report of Thompson and Appleman (28). These authors reported a single phosphodiesterase for cGMP in rat kidney which was found exclusively in the 20,000 x g supernatant fluid. They reported a Km of 9.25 µM cGMP, a value somewhat lower than ours. In most other tissues, however, they reported the Km for cGMP to be about 30 µM, which would be similar to the values we obtained. The reason for the high phosphodiesterase activity for cGMP in the neonatal rat kidney is not apparent from our studies, but could be secondary to increases in cGMP generation and the high cGMP levels previously reported by us (24). For example, cGMP has been shown to activate phosphodiesterase activity in several tissues (1).

By analogy with the action of cAMP on specific protein kinases a similar mechanism has been postulated for cGMP (13). Recently, cGMP dependent protein kinase has been demonstrated and partially characterized from a number of mammalian tissues (9,14,17,20,25,27,29). An intriguing observation on changes in the levels and ratios of cyclic-nucleotide-dependent protein kinases was made by Kuo in developing tissues of the guinea pig (15,16). In the lung and heart, the ratio of cGMP-dependent to cAMP-dependent enzyme activity was higher in the fetus than in the neonate and adult, suggesting that the events mediated by cGMP-dependent protein kinase may be particularly important during the early stage of development of these tissues. It was therefore of interest that cGMP-dependent protein kinase activity could be demonstrated by Sephadex chromatography in kidneys of newborn rats and not in the preparation from adult rats. These results together with our previous report of elevated cGMP levels in developing and hypertrophying kidneys of rats (24) support a role for cGMP during kidney development, though the exact role of cGMP and cGMP-related enzymes remains open to speculation.

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30. Send requests for reprints to: Dr. Detlef Schlondorff, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461.

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Table 1

Renal guanylate cyclase activity

	Cyclic GMP formed (pmol/min per mg protein) 7 day old	Adult	P
Homogenate	48.1 ± 1.6	29.6 ± 1.1	<0.001
Soluble	98.8 ± 3.5	68.7 ± 5.9	<0.005
Particulate	97.5 ± 2.0	31.6 ± 7.3	<0.001
	21 day old	Adult	P
Homogenate	34.0 ± 1.8	23.5 ± 2.7	<0.02
Soluble	88.5 ± 9.1	60.4 ± 4.5	N.S.
Particulate	34.1 ± 1.9	15.2 ± 0.5	<0.001

Kidneys from newborn and adult rats were prepared and guanylate cyclase activity was assayed in the homogenate, the 100,000 x g supernatant (soluble) and pellet (particulate) as described in Methods. Results represent the mean ± SEM of 4 rats in each group. P-value refers to group comparison between newborn and adult rats.

Table II

Phosphodiesterase activity for cGMP in the 16,000 x g supernatant of kidney homogenate from newborn and adult rats

Substrate Concentration	Cyclic GMP hydrolyzed (pmol/min per mg protein) 7 day old	P	21 day old	P	Adult
5 µM cGMP	1065 ± 73	<0.001	1265 ± 72	<0.001	407 ± 62
125 µM cGMP	7488 ± 831	<0.02	7674 ± 1120	<0.025	4042 ± 122

Kidneys from newborn and adult rats were prepared and assayed for phosphodiesterase as described in Methods. Values represent mean ± SEM of 4 rats in each group. P-value refers to group comparison between newborn and adult rats.

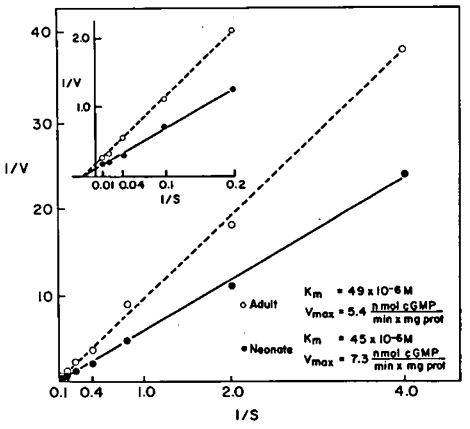

Fig. 1

Lineweaver-Burke plot of phosphodiesterase activities for cGMP in the 16,000 x g supernatant of kidneys from one adult and newborn (1 week old) rat. Kidneys were prepared and assayed as described in Methods. Each point represents the mean of a duplicate determination from one rat. The given values for Km and Vmax were calculated from the double reciprocal plot.

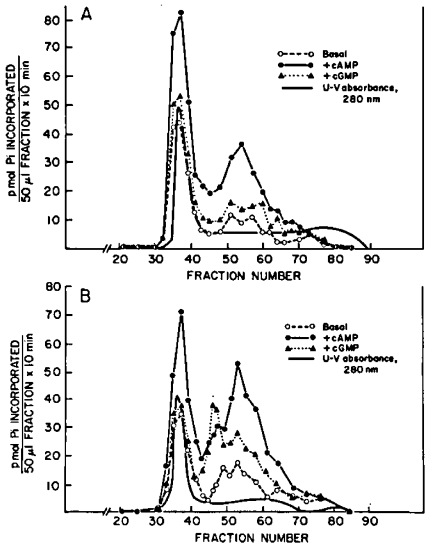

Fig. 2

Comparison of elution pattern of protein kinase of adult and 10 day old rat kidneys from Sephadex G200; results shown are from one of two similar experiments. The 30,000 x g supernatant solution from 1 gram of fresh kidney (2 rats for the adult and 10 for the young rats) was precipitated at pH 5.4 with 0.2 N acetic acid and the precipitate was resuspended in 2 ml of 50 mM potassium-phosphate buffer (pH 7) with 50 mM mercaptoethanol, charged onto a Sephadex G-200 column and eluted with the same buffer. Fractions of 2.5 ml were collected and 50 µl aliquots were assayed for protein kinase activity with or without cAMP and cGMP as described in Methods. Fig. 2A shows the pattern obtained from adult rats, while Fig. 2B shows the pattern obtained from 10 day old rats.

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