Biochimica et Biophysica Acta, 377 (1975) 463—472

BBA 67413

RENAL CORTEX GUANYLATE CYCLASE

PREFERENTIAL ENRICHMENT IN GLOMERULAR MEMBRANES

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(Received August 15th, 1974)

Summary

1. The localisation and some of the properties of rabbit kidney cortex guanylate cyclase (GTP pyrophosphatase lyase (cyclizing) EC 4.6.1.2) have been studied. Upon fractionation of dissociated renal cortex, guanylate cyclase activity was preferentially enriched in fractions of pure glomeruli, where its specific activity was 44.5 times that measured in tubular fragments. Most, if not all, of the glomerular activity was found to be firmly membrane-bound, whereas the guanylate cyclase activity of the tubules was mainly soluble. Therefore, particulate guanylate cyclase activity could serve as marker enzyme for kidney glomeruli.

2. All hormones or hormone-like agents tested were without effect on kidney guanylate cyclase activity. Triton X-100 stimulated both glomerular and tubular activity.

3. Considering the high cyclic GMP forming capacity of kidney glomeruli, part of the cyclic GMP found in urine might be synthetized locally in these structures.

Introduction

The interpretation of biochemical results obtained with kidney homogenates is rendered difficult by the extreme heterogeneity of kidney tissue, especially when hormonal effects are being studied. Even when slices of certain regions are being used, there are always structures with different functions present. We have devised a rapid technique to prepare pure glomeruli and

Abbreviation: EGTA, ethyleneglycol-bis-(2-aminoethyl ether) N,N'-tetraacetate.

tubular segments from rabbit kidney [1,2] in amounts suitable for biochemical studies.

Adenosine 3′,5′-monophosphate (cyclic AMP) is now recognized as the intracellular mediator of several hormonal effects on kidney tubules and a hormone-stimulated adenylate cyclase has been demonstrated in the kidney of several species [3-9].

Guanylate cyclase (GTP pyrophosphate-lyase (cyclizing) EC 4.6.1.2) and guanosine 3′,5′-monophosphate (cyclic GMP) have been identified in the mammalian kidney [10—12] but the function of kidney cyclic GMP is unclear at present. As a first step towards a better understanding of possible roles for kidney guanylate cyclase and cyclic GMP, we determined some properties of guanylate cyclase in purified glomeruli and tubular fragments from rabbit kidney.

Materials

For fractionation of dissociated renal cortex, the buffer consisted of 154 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 3.3 mM Tris · HCl buffer (pH 7.4), and will be referred to as Buffer A. The different fractions were homogenized in Buffer B, containing 10 mM Tris · HCl buffer, 50 mM NaCl, 0.4 mM ethyleneglycol-bis-(2-aminoethyl ether) *N,N′*-tetraacetate (EGTA) and 0.25 M sucrose (pH 7.4).

The following materials and reagents have been used with the source indicated in brackets. Cyclic GMP, cyclic AMP, GTP, creatine phosphate and creatine kinase (Boehringer, Mannheim, GFR). [α-^{32}P]GTP, spec. act. 2—8 Ci/mmol, cyclic [G-^{3}H]GMP, spec. act. 1—5 Ci/mmol, and Omnifluor (NEN, Dreieichenhain, GFR). Bovine serum albumine, cyclic nucleotide phosphodiesterase from beef heart lysine-vasopressin grade IV, parathyroid hormone as trichloroacetic acid-powder, carbachol as chloride salt (Sigma, St. Louis, MO). Hypertensin = angiotensin II amide 5-valine (Ciba-Geigy, Basel, Switzerland). Prostaglandin E_1 and $F_2\alpha$ were a generous gift of Dr John E. Pike of Upjohn CO. (Kalamazoo, Mich.). Al_2O_3, W200 neutral, (Woelm, Eschwege, G.F.R.). Silica gel-coated glass fiber sheets Chrom-AR 500 (Mallinckrodt, New York).

All other reagents were commercial preparations of the highest purity available.

Methods

Isolation of glomeruli and tubular segments

The kidney cortex from 1.5—2 kg rabbits was fractionated as described in detail elsewhere [1,2]. Briefly, the cortex of two kidneys was forced through a tissue press and incubated in 100 ml of Buffer A containing 0.05% collagenase at 37°C for several 20 min intervals. After each incubation period, the dissociated tissue was removed by filtering through nylon gauze and the residue further digested by addition of fresh collagenase. The collagenase and lysed cells were eliminated by repeated washing and the dissociated tissue, resuspended in Buffer A, was placed on a 3-step sucrose gradient (8.56, 58.6 and 75%) containing Buffer A throughout. The purified glomeruli sedimented at

the 58.6—75% interphase, whereas the tubular fragments were recovered from the 8.56—58.6% interphase. The tubular fraction was recentrifuged once more through 58.6% sucrose. Both fractions were very pure as judged from the morphology and the distribution of alkaline phosphatase [1,2], which can be regarded as a tubular marker enzyme [13, 14].

Preparation and fractionation of homogenates

The glomeruli and tubules recovered from the gradient were washed once with Buffer A and homogenized in a small volume of Buffer B. A portion of the renal cortex pulp and an aliquot of the dissociated tissue were also homogenized in Buffer B, to give the total cortex and digested cortex fractions. The volume of buffer was adjusted to give a protein concentration of around 15 mg/ml for total and digested cortex and tubules and 4 mg/ml for glomeruli. The homogenates could be stored at +4°C for at least 2 days without loss of guanylate cyclase activity.

Supernatants and particulate fractions were obtained by centrifuging homogenates at $9 \cdot 10^4 \times g$ for 1 h (4°C). The pellets were washed once with Buffer B and the supernatants combined. The washed pellets were taken up in the original volume of Buffer B.

Guanylate cyclase was assayed as previously described [15] with some minor modifications. The reaction medium contained: 15 mM creatine phosphate, 75 μg creatine kinase, cyclic [G-^{3}H]GMP (7 mM; $1 \cdot 10^4$ cpm), 8 mM $MnCl_2$, 60 mM Tris · HCl buffer (pH 7.5) and 0.04 ml enzyme with either 0.15—0.6 mg protein (total and digested cortex, tubules) or 0.02—0.15 mg protein (glomeruli) in a total volume of 0.1 ml. A preincubation without added substrate (5 min at 37°C) was found to increase guanylate cyclase activity in total cortex homogenates without affecting the activity in the other fractions and therefore carried out routinely. Reactions were initiated by addition of [α-^{32}P]GTP (0.185 mM, $1 \cdot 10^6$—$1.5 \cdot 10^6$ cpm) in 20 μl, and terminated, after 10 min at 37°C, by adding unlabelled GTP (250 nmol) and boiling for 3 min. The addition of a 10-fold excess of unlabelled GTP minimized the non-enzymatic formation of cyclic [^{32}P]GMP. Then, 0.1 ml ice-cold 0.06 M Tris · HCl buffer (pH 7.5) was added and the denatured protein pelleted. Cyclic GMP was separated from other labelled compounds by chromatography on Al_2O_3 columns. 200 μl of the supernatants was transferred to columns (1.5 g dry Al_2O_3) and the columns washed with 2 ml 60 mM Tris · HCl buffer (pH 7.5). Cyclic GMP was subsequently eluted with 2 ml 600 mM Tris · HCl buffer (pH 7.5) directly into counting vials containing 13 ml of a fluor cocktail (toluene/Triton X-100/omnifluor (21 : 11 : 23, by wt). Radioactivity was counted in an Intertechnique SL 30 liquid-scintillation counting system with the present ^{3}H/^{32}P double isotope counting window.

Boiled enzyme was used to determine the blanks, which did not exceed 0.02% of the total counts. The overall recovery of trace amounts of cyclic [^{3}H]GMP incubated and processed with these blanks was 70—76%. The recovery of cyclic [^{3}H]GMP incubated with active enzyme preparations was much lower, presumably due to the action of cyclic nucleotide phosphodiesterase. Therefore, unlabelled cyclic GMP was added to the incubation mixture to reduce degradation of cyclic [^{32}P]GMP by phosphodiesterases and the extent

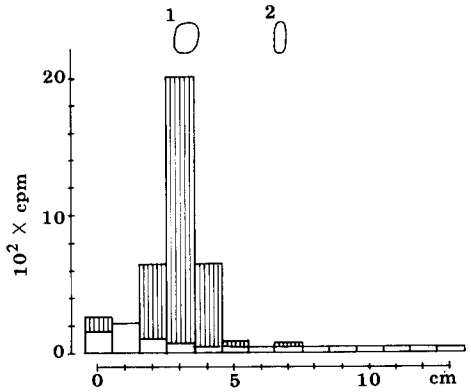


Fig. 1. Chromatography of cyclic [^{32}P]GMP on glass fiber sheets. Aliquots of glomerular membrane fractions were assayed for guanylate cyclase activity as described, but the presence of [α-^{32}P]GTP with a 10 times increased specific activity. 100 μl of the cyclic GMP containing eluate of the Al_2O_3 columns were spotted onto Chrom AR-500 sheets as a streak and chromatographed ascendingly with Solvent I. Authentic cyclic AMP was chromatographed on the same sheet. The cyclic nucleotides were visualized under ultraviolet light (254 nm). Then, 1-cm bands were cut out, dispersed in 1 ml 0.5 M NH_4OH and, after addition of 13 ml of the fluor cocktail, the ^{32}P radioactivity determined. The white columns represent the radioactivity recovered in the corresponding bands when the GTP substrate had been incubated with boiled enzyme and the hatched columns the increase found due to active enzyme. 1, cyclic GMP; 2, cyclic AMP.

of the degradation was checked routinely by addition of cyclic [^{3}H]GMP. The hydrolysis of cyclic GMP during the incubation period was kept below 10% in all fractions. With the aid of double isotope counting, all results were corrected for recovery of cyclic GMP.

To identify the radioactive products as cyclic GMP, the 2 ml 600 mM Tris · HCl buffer eluate from the Al_2O_3 column containing the cyclic [^{3}H]GMP added and the cyclic [^{32}P]GMP formed was analysed as follows:

(1) The eluate was applied to a second Al_2O_3 column with or without prior treatment with cyclic nucleotide phosphodiesterase. In samples not treated with phosphodiesterase, over 90% of the ^{32}P and ^{3}H was found in the 2 ml 600 mM Tris · HCl buffer eluate of the second column. Less than 5% of the ^{32}P and ^{3}H from treated samples appeared in this fraction.

(2) Aliquots of the Al_2O_3 eluate were chromatographed on Chrom AR sheets using either 2-propanol/ethyl acetate/11.8 M NH_4OH (55 : 29 : 17, by vol.; Solvent I) or *tert*-butanol/ethyl acetate/8.2 M NH_4OH (111 : 78 : 111 by vol.) as described by Woods and Waitzman [16]. The ^{32}P recovered chromatographed with authentic cyclic GMP in both solvents. The radioactivity associated with the cyclic AMP spot did not exceed the blank values. The results for Solvent I are shown in Fig. 1.

In control experiments, carried out for all fractions, the GTP regenerating system was excluded from the incubation mixture. The measured activity was maximally reduced by only 20% (for glomerular membranes). It was therefore concluded, that the regenerating system was sufficient to maintain the GTP concentration throughout the incubation period.

Results

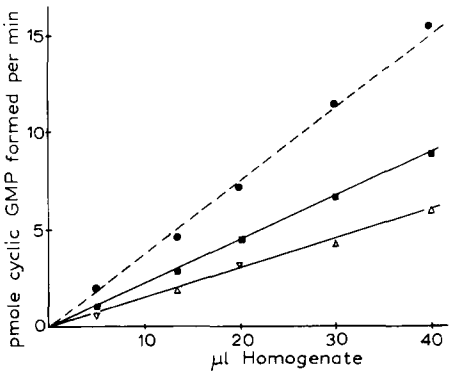
Basic conditions for the formation of cyclic GMP in homogenates of renal cortex fractions

Under the conditions used in our experiments, the accumulation of the cyclic nucleotide was a linear function of the amount of homogenate up to concentrations equivalent to 0.13 mg (glomeruli) or 0.65 mg protein (all other fractions) (Fig. 2). At higher homogenate concentrations, the reaction became non-linear due to degradation of a measurable fraction of the cyclic GMP by cyclic nucleotide phosphodiesterase. The cyclic ^{32}P formation was linear with time up to 15 min of incubation. Therefore, 10 min incubations were used routinely.

Guanylate cyclase activity in all fractions depended on the presence of Mn^{2+} as described for the rat kidney enzyme [12]. Maximum activities were attained in the presence of 8 mM $MnCl_2$ in purified glomeruli and total homogenates, higher concentrations being inhibitory (Fig. 3). Mg^{2+}, even in higher concentrations, could not replace Mn^{2+} as divalent cation: 1% of the maximum activity was measured in the absence of added divalent cation or in the presence of 10 mM $MgCl_2$. In earlier studies, however, a partial recovery of enzyme activity in the presence of high Mg^{2+} has been reported in kidney homogenates [12]; the discrepancy might be caused by the presence of EGTA in our homogenisation medium. Ca^{2+} was ineffective in replacing Mn^{2+}. When added to the assay mixture containing 8 mM $MnCl_2$ in concentrations from 0.125 (equimolar to the EGTA present) to 1 mM, no effect on guanylate cyclase activity was observed.

Localisation of kidney cortex guanylate cyclase

Guanylate cyclase specific activities of the different fractions obtained from rabbit renal cortex are given in Table I. The most interesting feature of



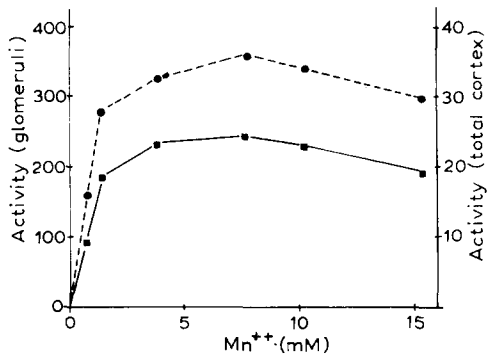


Fig. 2. Formation of cyclic [^{32}P]GMP in homogenates of total cortex (■- - - - -■), glomerular (●- - - - -●) and tubular (△- - - - -△). The protein concentration of the homogenates was 3.2 mg/ml for the glomerular and 16 mg/ml for the other fractions. Increasing amounts of the homogenates were added to the test.

Fig. 3. Formation of cyclic [^{32}P]GMP in homogenates of total cortex (■- - - - -■) and glomerular (●- - - - -●) fractions as a function of Mn^{2+} concentration. Guanylate cyclase activity is expressed as pmol cyclic GMP formed/min per mg protein.

TABLE I

GUANYLATE CYCLASE ACTIVITY IN PURIFIED GLOMERULI AND TUBULES FROM RABBIT KIDNEY

Guanylate cyclase assays were performed using rabbit renal cortex preparations as described. Cyclic GMP formation is expressed as pmol/min per mg protein, the mean ± S.E. is given for 8 experiments; $P < 0.01$ for the differences between all fraction.

Fraction	Activity	Enrichment
Homogenate	28.3 ± 2.3	1
Digested homogenate	20.7 ± 2.0	0.73
Glomerular homogenate	414 ± 40	14.6
Tubular homogenate	9 ± 0.6	0.32

guanylate cyclase distribution was its remarkable concentration in purified glomeruli, where guanylate cyclase specific activity was more than 40 times higher than in purified tubular segments.

The technique used for tissue disruption and isolation of kidney structures makes losses of material inevitable (Table II), mainly during the digestion procedure and the subsequent washings. The recovery of guanylate cyclase activity paralleled reasonably well the protein recovery. If allowance was made for protein losses, then, 70% of the total activity was recovered in the highly purified glomeruli and tubules. 62.5% of the guanylate cyclase activity recovered with the pure fractions was found in the glomeruli. The possibility, however, cannot be ruled out that preferentially tubular material has been lost during the isolation procedure. But there seems to be a preferential loss of glomeruli since we recovered 1% of the total protein with the glomeruli and 33% with the tubules, whereas for intact kidney cortex, a ratio glomeruli: tubules of 1 : 8.5 can be estimated [17].

Separation of soluble and particulate fractions from the glomerular and tubular homogenates revealed striking differences in the distribution of guanylate cyclase. Whereas most of the tubular activity was recovered in a $9 \cdot 10^4 \times g$ supernatant, over 80% of the cyclic GMP forming capacity of the glomeruli was found to be associated with washed particulate fractions (Table III). The particulate fraction prepared from glomeruli was treated in various ways to release loosely bound or adsorbed protein into the supernatant. When the washed pellet obtained in isotonic buffer was homogenized in 10 mM Tris ·

TABLE II

RECOVERY OF PROTEIN AND GUANYLATE CYCLASE FROM KIDNEY CORTEX UPON ISOLATION OF GLOMERULI AND TUBULAR FRAGMENTS

The values are given as the mean ± S.E. for 5 experiments. Guanylate cyclase activity is expressed as nmol cyclic GMP formed/ per min.

Fraction	mg protein per fraction	% recovery	Activity per fraction	% recovery
Total homogenate	909 ± 79	100	25.4 ± 1.6	100
Digested homogenate	421 ± 29	46	7.8 ± 0.74	31
Glomeruli	9.4 ± 1.6	1.0	3.7 ± 0.38	15
Tubules	304 ± 25	33	2.3 ± 0.13	9.0

TABLE III

FORMATION OF CYCLIC GMP IN SOLUBLE AND PARTICULATE FRACTIONS FROM ISOLATED RABBIT KIDNEY STRUCTURES

Homogenates of total kidney cortex, purified glomeruli or tubular segments were centrifuged at $9 \cdot 10^4 \times g$ for 1 h. The pellets were washed once and the supernatants combined. Guanylate cyclase activity is expressed as pmol/min per mg protein, the results for two experiments are shown.

Fraction	Glomeruli		Tubules	
	Activity	% recovered	Activity	% recovered
Homogenate	285, 310	—	7.7, 7.6	—
Supernatant	88, 104	14, 12	17.1, 10.2	73, 65
Pellet	464, 570	86, 88	5.0, 6.2	27, 35

* % of recovered activity. % total recovery: 97 and 99% for glomerular and 143 and 127% for tubular homogenates. Protein recovery was 95 and 97%.

HCl buffer (pH 7.5) with or without 0.4 mM EGTA, frozen and thawed several times and recentrifuged ($9 \cdot 10^4 \times g$ for 1 h), no guanylate cyclase activity was detected in the supernatant. In another series of experiments, the particulate fraction from glomeruli was homogenised in 0.5 M NaCl buffered with 10 mM Tris · HCl buffer (pH 7.5) and recentrifuged. Only 65% of the total activity were recovered, but 90—94% of the recovered activity was still associated with the membranes.

Effect of various agents on cyclic GMP formation

Prostaglandin $F_2\alpha$ (10^{-7}—10^{-5} M), prostaglandin E_1 (10^{-5} M) hypertensin (10^{-8}—10^{-5} M) and carbachol (10^{-8}—10^{-4} M), when added to the assay mixture in the concentration range indicated, were without effect on cyclic GMP formation in glomerular or tubular fractions. Also agents known to stimulate mammalian kidney adenylate cyclase, vasopressin [18,19], parathyroid hormone [18,19] and fluoride [19] were without effect on guanylate cyclase activity of tubular homogenates and particulate fractions from glomeruli (Table IV). The only substance which was found to stimulate cyclic GMP formation was the non-ionic detergent Triton X-100 (Table V). Interestingly, approximately the same stimulation was observed in glomerular homogenates, where the enzyme appeared to be membranebound, and in tubular homogenates, where most of

TABLE IV

LACK OF EFFECT OF VARIOUS AGENTS ON GUANYLATE CYCLASE ACTIVITY IN TUBULES AND IN GLOMERULAR MEMBRANES

Vasopressin (10^{-6} M), parathyroid hormone (50 μg/ml) and NaF (10 mM) were added to the usual incubation mixture and preincubated in the presence of enzyme at 37°C for 5 min before adding substrate. The mean of two determinations is given; the duplicates varied less than ± 6%.

Fraction	Cyclic GMP formation* (pmol/min per mg protein)			
	Control	Vasopressin	Parathyroid hormone	F^-
Tubules	7.8	8.4	8.1	7.6
Glomerular membranes	464	445	485	477

TABLE V

THE EFFECT OF TRITON X-100 ON GUANYLATE CYCLASE ACTIVITY IN DIFFERENT FRACTIONS FROM RENAL CORTEX

Triton X-100 was added to the usual incubation mixture in a concentration of 1.5%. Before adding substrate, the mixture was incubated for 5 min at 37°C. Guanylate cyclase activity is expressed as percent of the control without added Triton X-100. The mean and the range is given for 4 experiments.

Fraction	Cyclic GMP formation (% of control)
Glomeruli	465 (400 — 615)
Tubules	383 (340 — 400)
Tubules, soluble fraction	258 (240 — 272)

the activity was soluble. Indeed, also the soluble enzyme prepared from tubular homogenates was stimulated by Triton X-100.

Discussion

Several studies have demonstrated the occurrence of guanylate cyclase activity in cell-free preparations of rat total kidney [10,12] and kidney cortex [17]. We have shown in the present study that rabbit cortex guanylate cyclase activity was preferentially enriched in isolated glomeruli, the specific activity in tubular fragments being very low. About 60% of the activity recovered were found associated with the glomeruli, which make up not more than 8% of the mass of rabbit kidney cortex [17]. Most of the glomerular activity appeared to be firmly membrane bound. Guanylate cyclase specific activity of these membranes exceeded the highest value in any subcellular fraction of rat brain [20], especially rich in guanylate cyclase [10], by a factor of two. These results are in essential agreement with those of Schultz et al. [18], who found higher guanylate cyclase activities in a particulate fraction than in the cytosol of rat kidney cortex. Upon subcellular fractionation of tubular homogenates, very little activity was recovered with the particulate fraction. Therefore, the enzyme detected in tubular segments cannot be due to the presence of contaminating glomerular cells, but should represent intrinsic tubular activity. However, taking into account the large differences in specific activity between the two kidney structures, guanylate cyclase, especially its membrane-bound form, could still serve as a marker enzyme for glomeruli.

The manganese dependance of guanylate cyclase activity was the same in glomeruli and total kidney cortex and similar to that of guanylate cyclase of various sources [10,12,20,21]. We did not find a stimulatory action of Ca^{2+} as reported for kidney guanylate cyclase [12], but the presence of Ca^{2+} during the isolation procedure and the subsequent addition of EGTA could have obscured such an effect.

Most attempts to demonstrate a hormone-dependant activation of guanylate cyclase in vitro have been unsuccessful [18,21,22]. In our hands, a variety of hormones and hormone-like agents known either to activate adenylate cyclase from kidney and other sources [18,19,23] or to increase cyclic GMP levels in intact tissue preparations [22] did not stimulate guanylate cyclase activity. Even, when added to cell-free preparations of glomeruli, where

most of the activity was firmly membrane-bound and could possibly be coupled to a receptor-system, these substances were without effect.

Addition of the non-ionic detergent Triton X-100 increased guanylate cyclase activity of glomerular and tubular homogenates 4-fold. The increase of the tubular activity (recovered mostly in a $9 \cdot 10^4 \times g$ supernatant) can best be explained by activation of soluble enzyme. Indeed, soluble guanylate cyclase prepared from tubular fragments, was activated by the detergent. The interesting, albeit unexplained, stimulation of soluble enzyme activity by Triton X-100 has already been reported for the soluble guanylate cyclase from rat lung [24].

Cyclic GMP is excreted in rat [11,25], dog [11] and human [26] urine in relatively important amounts. Whereas Goldberg et al. [11] suggested, on rather indirect evidence, that at least part of the cyclic GMP in dog urine was synthesized in the kidney, the results of Broadus et al. [26] seem to indicate plasma as the source of the cyclic GMP excreted by healthy human subjects. Experiments are now under way to determine, whether part of the cyclic GMP excreted in rabbit urine might be produced by the kidney, possibly in the glomeruli.

Although cyclic GMP was identified as a natural constituent of kidney tissue, no functional role can yet be attributed to kidney cyclic GMP. The high and specifically membrane-associated cyclic GMP forming capacity of isolated glomeruli indicates that this cyclic nucleotide might be implicated in glomerular rather than in tubular function.

Acknowledgements

We are grateful to Ms J.M. Krill and M.O. Revel for their skilled technical assistance.

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