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Properties and Subcellular Distribution of Guanylate Cyclase Activity in Rat Renal Medulla: Correlation with Tissue Content of Guanosine 3′,5′-Monophosphate†

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ABSTRACT: The properties of the guanylate cyclase systems of outer and inner medulla of rat kidney were examined and compared with those of the renal cortex. A gradation in steady-state cyclic guanosine 3′,5′-monophosphate (cGMP) levels was observed in incubated slices of these tissues (inner medulla > outer medulla > cortex). This correlated with the proportion of total guanylate cyclase activity in the 100 000*g* particulate fraction of each tissue, but was discordant with the relative activities of guanylate cyclase (highest in cortex) and of cGMP-phosphodiesterase (lowest in cortex) in whole tissue homogenates. Soluble guanylate cyclase of cortex and inner medulla exhibited typical Michaelis–Menten kinetics with an apparent K_m for MnGTP of 0.11 mM, while the particulate enzyme from inner medulla exhibited apparent positive cooperative behavior and a decreased dependence on Mn^{2+}. Thus, the particulate enzyme could play a key role in regulating cGMP levels in the intact cell where Mn^{2+} concentrations are low. The soluble and particulate enzymes from inner medulla were further distinguished by their responses to several test agents. The soluble enzyme was activated by Ca^{2+}, NaN_3, $NaNO_2$, and phenylhydrazine, whereas particulate activity was inhibited by Ca^{2+} and was unresponsive to the latter agents. In the presence of $NaNO_2$, Mn^{2+} requirement of the soluble enzyme was reduced and equivalent to that of the particulate preparation. Moreover, relative responsiveness of the soluble enzyme to $NaNO_2$ was potentiated when Mg^{2+} replaced Mn^{2+} as the sole divalent cation. These changes in metal requirements may be involved in the action of $NaNO_2$ to increase cGMP in intact kidney. Soluble guanylate cyclase of cortex was clearly more responsive to stimulation by NaN_3, $NaNO_2$, and phenylhydrazine than was soluble activity from either medullary tissue. The effectiveness of the agonists on soluble activity from outer and inner medulla could also be distinguished. Accordingly, regulation and properties of soluble guanylate cyclase, as well as subcellular enzyme distribution, are distinct in the three regions of the kidney.

Cyclic guanosine 3′,5′-monophosphate (cGMP[1]) has been implicated as an intracellular modulator of several important physiological processes (Berridge, 1975) and may be involved in the regulation of renal metabolic and excretory functions (Goodman et al., 1972; DeRubertis et al., 1976). Previous studies from our laboratory have described the properties of the guanylate cyclase–cGMP system of rat renal cortex. In this tissue, both Ca^{2+}-dependent (DeRubertis and Craven, 1976a) and independent mechanisms (DeRubertis and Craven, 1976b) exist for modulation of cGMP levels, and guanylate cyclase activity is found predominantly in the soluble fraction of cell homogenates. The inner medulla of kidney is known to be unique in several of its anatomical, functional, and metabolic characteristics (Rhodin, 1958; Kean et al., 1961, 1962; Bernanke and Epstein, 1965; Sternberg et al., 1956; Lee et al., 1962; Rennie et al., 1958; Aperia and Leebow, 1964; Cohen and Barac-Nieto, 1973). Preliminary observations (Craven and DeRubertis, 1976a) indicated that these differences extended to the properties of the guanylate cyclase–cGMP system of this tissue compared with cortex or outer medulla. Specifically, basal cGMP levels in incubated slices of inner medulla are consistently higher than those of cortex or outer medulla. Basal cGMP did not correlate with the relative activities of guanylate cyclase (highest in cortex) or cGMP-phosphodiesterase (lowest in cortex) found in whole homogenates of the three tissues. Data from other tissues have suggested that the particulate form of guanylate cyclase may be a key determinant of cGMP economy in the intact cell due to its distinct properties (Kimura and Murad, 1974, 1975a,b;

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[1] Abbreviations used are: cGMP, cyclic guanosine 3′,5′-monophosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; SE, standard error; SEM, standard error of the mean; Tris, tris(hydroxymethyl)aminomethane; ip, intraperitoneally.

Garbers et al., 1975; Chrisman et al., 1975; Gray and Drummond, 1976; Gray et al., 1976). Accordingly, the present studies were conducted to define the subcellular distribution of guanylate cyclase and cGMP-phosphodiesterase activities in different regions of the kidney, and to characterize further the properties of the guanylate cyclase–cGMP system of inner medulla.

Materials and Methods

3-Isobutyl-1-methylxanthine was purchased from Aldrich Chemical Co., Milwaukee, Wis.; [^{14}C]guanosine (specific activity 40–60 mCi/mmol), [α-^{32}P]GTP (specific acitivity 20 Ci/mmol), and [^{3}H]cGMP[1] (specific activity 10 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Sources of all other materials have previously been reported (DeRubertis and Craven, 1976a,b; DeRubertis et al., 1976).

Male Sprague-Dawley rats (300–350 g) were anesthetized with pentobarbital (5 mg/100 g of body weight ip) and the kidneys quickly excised and placed in 0.85% NaCl at 0–4 °C. Six cortical and three medullary slices were prepared from each kidney using a Stadie-Riggs microtome to ensure minimal cross-contamination of cortex and medulla. Cortex and outer and inner medulla were further separated by careful dissection of each slice on a filter paper moistened with cold saline. Approximately 25 mg of inner medulla, 40 mg of outer medulla, and 600 mg of cortex could be obtained from a single rat kidney.

Assessment of Tissue cGMP Levels. Slices of renal cortex and outer and inner medulla were incubated at 37 °C for 20 min in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) containing 1 mg/ml of glucose and bovine serum albumin in the presence or absence of 2 mM 3-isobutyl-1-methylxanthine as previously described in detail (DeRubertis and Craven, 1976a,b; DeRubertis et al., 1976). At the end of the 20-min incubation, tissue slices were extracted and assayed for cGMP by radioimmunoassay (Steiner et al., 1972). The details of these procedures and those used to verify the authenticity of cGMP determinations in tissue extracts have previously been described (DeRubertis and Craven, 1976a,b; DeRubertis et al., 1976).

Preparation of Tissue for Enzyme Assay. Tissue slices were homogenized in 0.25 M sucrose–5 mM Tris, pH 7.4 (125 mg/4 ml), using a ground-glass homogenizer. Slices were homogenized without prior incubation except where indicated in the Results. They were either assayed directly or fractionated as indicated in the legends to the tables and figures. All particulate fractions were washed with twice the original volume of the sucrose Tris buffer. The particulate fractions of outer and inner renal medulla were resuspended in the same volume as the original homogenate, while those from the renal cortex were resuspended in one-half the original volume of the homogenates Samples were diluted, where necessary, to achieve linear kinetics and kept at 0–4 °C until addition to the enzyme reaction mixtures.

cGMP-Phosphodiesterase Activity. cGMP-phosphodiesterase activities of whole homogenates and soluble and particulate fractions of renal cortex and outer and inner medulla were assayed as previously reported (DeRubertis and Craven, 1976b). The procedure of Thompson and Appleman (1971) was employed, with one modification [^{14}C]Guanosine (3000 cpm) was added just before the treatment of samples with snake venom, in order to assess loss of guanosine through absorption to AG1-X2 resin (Boudreau and Drummond, 1975) This loss was consistently 47–52% and the results were corrected accordingly. cGMP hydrolysis was determined at a substrate concentration of 0.1 μM with sufficient tissue added to destroy 20% of the substrate. The hydrolysis of cGMP was linear with time for at least 5 min at 30 °C. Results are expressed as pmol of cGMP hydrolyzed per min per mg of protein.

Guanylate Cyclase Activity. Guanylate cyclase was determined as previously described in detail (Craven and DeRubertis, 1976b). Standard reaction mixtures contained, at final concentration, 50 mM Tris (pH 7.6), 10 mM theophylline, 2.7 mM cGMP, 4 mM $MnCl_2$, 15 mM creatine phosphate, 37 μg of creatine kinase (155 units/mg), and 1 mM [α-^{32}P]GTP (2×10^6 cpm) (specific activity 20 Ci/mmol). The cGMP formed was isolated from the reaction mixtures by sequential chromatography on Dowex 50 AG1-X4 and neutral alumina (Craven and DeRubertis, 1976b). Blank counting rates were approximately 50 cpm, with all experimental rates at least twice this blank. Product formation was linear with time under all conditions described for at least 10 min at 37 °C and with respect to added protein in homogenates, particulate (20 to 100 μg), and soluble (10 to 50 μg) fractions. Protein was determined by the Lowry method (Lowry et al., 1951). Statistical significance of differences between mean values was assessed by Student's t test for unpaired data. The results presented are the means ± SEM of duplicate or triplicate determinations from the number of experiments indicated in the footnotes to the tables. When results from single representative experiments are shown, these studies were repeated at least twice.

Results

After a 20-min incubation at 37 °C in Krebs–Ringer bicarbonate buffer, the cGMP content of renal cortex (0.27 ± 0.03 pmol/mg of protein) and outer medulla (0.45 ± 0.04) and inner medulla (0.66 ± 0.07) differed significantly (mean ± SEM of 18 slices pooled from six experiments, with $p < 0.005$). The presence of 2 mM 3-isobutyl-1-methylxanthine in the incubates markedly potentiated the differences among the three tissues (cortex, 1.59 ± 0.52 pmol/mg of protein; outer medulla, 3.96 ± 0.44; inner medulla, 6.63 ± 0.07). The relative levels of cGMP in the tissues did not change appreciably after an additional 120 min of incubation, and accumulation of cGMP in the media was also significantly greater in the medullary than in the cortical incubates (not shown). cGMP-phosphodiesterase activity (mean ± SE of determinations pooled from six separate experiments) was significantly greater in whole homogenates of outer medulla (264 ± 33 pmol of cGMP hydrolyzed per min per mg of protein) and inner medulla (315 ± 39) than in cortex (59 ± 7), with $p < 0.005$. These latter results and the effects of 3-isobutyl-1-methylxanthine suggested that higher levels of cGMP in medullary tissues compared with cortex might be due to an increased rate of cGMP synthesis, which was partially offset by an increased rate of cGMP degradation in the medulla.

However, when determined in whole homogenates of the three tissues, guanylate cyclase activities of medullary preparations were significantly less than that of cortex (Table I). Prior incubation of slices of the three tissues for 20 or 120 min at 37 °C in Krebs buffer did not change the relative activities of guanylate cyclase (not shown). As summarized in Table I, 19% of the total guanylate cyclase activity in renal cortex, 44% of that in outer medulla, and 63% of that in inner medulla were recovered in the 100 000g particulate fractions in the absence of prior detergent treatment. After incubation with 1% (v/v) Triton X-100, guanylate cyclase activity of whole homogenates increased 2-fold in cortex, 5.2-fold in outer medulla, and

TABLE I: Guanylate Cyclase Activities of Rat Renal Cortex and Outer and Inner Medulla in the Presence and Absence of Triton X-100.[a]

	Whole Homogenate		100 000*g* Soluble Fraction		100 000*g* Particulate Fraction	
Triton X-100	−	+	−	+	−	+
	Total Act. (pmol min^{-1} (mg $protein^{-1}$) in homogenate)					
Cortex	62 ± 5	125 ± 10[d]	45 ± 4	54 ± 4	11 ± 1	62 ± 5[d]
Outer medulla	34 ± 4[b]	177 ± 17[b,d]	18 ± 2[b]	31 ± 3[b,d]	14 ± 1	128 ± 9[b,d]
Inner medulla	43 ± 4[b]	322 ± 28[b-d]	15 ± 2[b]	7 ± 0.6[b-d]	25 ± 2[b,c]	290 ± 26[b-d]
	Spec Act. (pmol min^{-1} (mg $protein^{-1}$) in fraction)					
Cortex	−	−	146 ± 11	175 ± 12	19 ± 2	108 ± 9[d]
Outer medulla	−	−	79 ± 6[b]	136 ± 11[b,d]	32 ± 2[b]	294 ± 25[b,d]
Inner medulla	−	−	45 ± 3[b,c]	22 ± 2[b-d]	58 ± 4[b,c]	675 ± 51[b-d]

[a] Renal cortex and outer and inner medulla were homogenized in 0.25 M sucrose, 5 mM Tris, pH 7.4 (125 mg of tissue/4 ml). A 3-ml aliquot was centrifuged for 1 h at 100 000*g*. The pellet was washed once in 6 ml of buffer, recentrifuged, and resuspended in 3 (inner and outer medulla) or 1.5 ml (cortex). A 0.5-ml aliquot of each fraction was treated for 1 h at 0–5 °C with 1% (v/v) Triton X-100 before assay. Samples (± Triton X-100) were assayed for 7 min at 37 °C using a standard reaction mixture. Total activity is expressed as pmol min^{-1} (mg $protein)^{-1}$ in the original homogenate (15.3 mg of cortical, 10.2 mg of outer, and 9.9 mg of inner medullary protein/4 ml of homogenate) Specific activity is expressed as pmol min^{-1} (mg $protein)^{-1}$ in the fraction studied. Recovery of activity from the isolated fractions was 90–95% of that in the whole homogenate. Each value represents the mean ± SEM of duplicate assays from four separate experiments. [b] p at least < 0.01 compared with the corresponding value in the cortex. [c] $p < 0.005$ compared with the corresponding value in the outer medulla. [d] $p < 0.005$ compared with the corresponding value in the absence of Triton X-100.

7.4-fold in inner medulla, with total activity highest in the latter tissue. When homogenates were centrifuged at 100 000*g* and the fractions then treated with Triton X-100, guanylate cyclase activity in each of the pellets was increased 6- to 12-fold (Table I). By contrast, activity in the soluble fraction was enhanced only slightly or, in the case of the inner medulla, was inhibited by 51%. Thus, the high guanylate cyclase activity observed in whole homogenates of inner medulla after treatment with detergent was attributable to the predominance of particulate guanylate cyclase in this tissue. After exposure of fractions to Triton X-100, the proportion of particulate enzyme was increased to 53% in cortex, 80% in outer medulla, and 97% in inner medulla. As also shown in Table I, the specific activity of particulate guanylate cyclase in the presence or absence of detergent was greatest in inner medulla. Thus, the high total activity of the particulate enzyme from inner medulla was not a simple function of relative subcellular protein distribution in this region of the kidney compared with the cortex and outer medulla. Moreover, in all three tissues, cGMP-phosphodiesterase activity was found predominantly (89% or greater) in the 100 000*g* soluble cell fraction (not shown).

Figure 1 compares the effects of varying the concentration of total $MnCl_2$ from 0.5 to 9 mM on the guanylate cyclase activities in the 100 000*g* soluble and particulate fractions of renal inner medulla in the absence of Triton. The enzyme in the particulate fraction was less dependent on the availability of Mn^{2+} in excess of GTP, as shown by the changes in activity between 0.5 and 2 mM total $MnCl_2$ (1 mM GTP). Further, the optimal concentration of total $MnCl_2$ with 1 mM GTP was 2 mM for the particulate and 4 mM for the soluble enzyme. Analogous curves were obtained in three separate experiments, with the SEM of duplicate determinations in each study 5% or less. As also shown in Figure 1, $CaCl_2$ inhibits the activity of the particulate enzyme and activates the soluble enzyme. These effects of Ca^{2+} are more clearly evident at concentrations of $MnCl_2$ less than GTP. With 1 mM GTP and 0.75 mM $MnCl_2$ in the reaction mixtures, mean soluble activity from determinations pooled from three separate experiments was 15 ± 1 pmol of cGMP min^{-1} (mg $protein)^{-1}$ in the absence

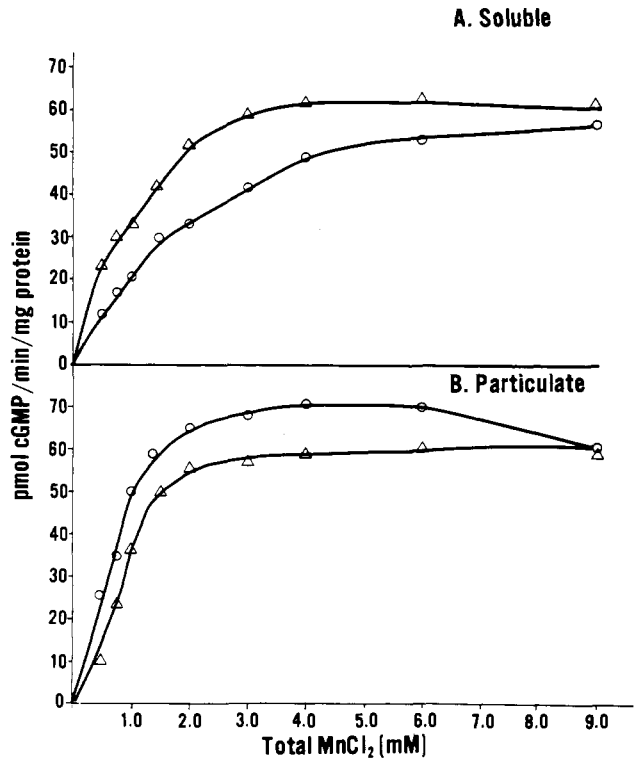

FIGURE 1: The effect of varying the total $mnCl_2$ concentration from 0.5 to 9.0 mM, in the presence (△) and absence (○) of 5.0 mM $CaCl_2$, on the guanylate cyclase activities of 100 000*g* soluble (A) and particulate (B) fractions of renal inner medulla. The GTP concentration was held constant at 1 mM and no detergent was used in the particulate preparation. The results presented are the means of duplicate assays from the same experiment repeated twice.

and 30 ± 2 pmol of cGMP min^{-1} (mg $protein)^{-1}$ in the presence of 5 mM Ca^{2+}, with $p < 0.005$. Under identical conditions, mean particulate activity was 35 ± 3 in the absence and 23 ± 2 pmol cGMP min^{-1} (mg $protein)^{-1}$ in the presence of 5 mM Ca^{2+}, with $p < 0.01$. The Triton solubilized particulate

TABLE II: The Effects of Various Agents on Guanylate Cyclase Activity in the 100 000*g* Soluble and Particulate Fractions of Renal Cortex and Outer and Inner Medulla.[a]

	Guanylate Cyclase Act. (pmol min^{-1} (mg $protein^{-1}$) in the homogenate)											
	Whole Homogenate				100 000*g* Soluble Fraction				100 000*g* Particulate Fraction			
Test Agent	—	NaN_3 10 mM	$NaNO_2$ 10 mM	C_6H_5NHN-H_2 1 mM	—	NaN_3 10 mM	$NaNO_2$ 10 mM	C_6H_5NHN-H_2 1 mM	—	NaN_3 10 mM	$NaNO_2$ 10 mM	C_6H_5NHN-H_2 1 mM
Cortex	55 ±2	1046[b] ±51	964[b] ±38	439[b] ±18	38 ±2	912[b] ±41	874[b] ±38	392[b] ±18	12 ±0.5	51[b] ±2	13 ±0.5	12 ±0.6
Outer medulla	41 ±2	68[c] ±2	67[c] ±2	166[b] ±7	23 ±1	43[c] ±3	45[c] ±2	138[b] ±5	15 ±0.6	17 ±0.9	14 ±0.6	15 ±0.7
Inner medulla	48 ±3	71[c] ±3	102[c] ±4	95[c] ±4	17 ±0.7	36[c] ±1.5	63[b] ±3	53[b] ±2	27 ±1	26 ±2	27 ±2	33 ±3

[a] Renal cortex and outer and inner medulla were homogenized in 0.25 M sucrose, 5 mM Tris, pH 7.4 (125 mg of tissue/4 ml). A 3-ml aliquot was centrifuged at 100 000*g*. The pellets were washed once in 6 ml of buffer, recentrifuged, and resuspended in 3 (inner and outer medulla) or 1.5 ml (cortex) for assay. Test agents were present in the reaction mixtures at the final concentrations indicated. These concentrations gave maximal increases in enzyme activity. Activity is expressed as total pmol min^{-1} (mg protein)$^{-1}$ in the original homogenate. The results shown are the means ± SEM of duplicate assays from three separate experiments. [b] p at least < 0.005 vs. corresponding control. [c] p at least < 0.05 vs. corresponding control.

enzyme of inner medulla was also significantly inhibited by Ca^{2+}.

Figure 2 shows a double-reciprocal plot comparing the effects of varying the concentration of MnGTP at a constant excess Mn^{2+} concentration[2] of 3 mM on the guanylate cyclase activity of 100 000*g* soluble and particulate fractions of renal inner medulla in the absence of Triton. The soluble enzyme exhibited typical Michaelis-Menten kinetics with an apparent K_m for MnGTP of 0.11 ± 0.01 mM (mean ± SEM), when calculated from data pooled from three separate studies. The plot for the particulate enzyme was concave upward (Figure 2), suggesting the presence of more than one positively interacting metal nucleotide binding site or enzyme heterogeneity. The slope of the Hill plot for the particulate enzyme (Figure 2, insert) was 1.33 ± 0.05 with an $S_{0.5}$ of 0.48 ± 0.02 mM, when calculated from data pooled from three separate studies. By contrast, the slope of the Hill plot for the soluble enzyme was 1.02 ± 0.05. Triton X-100 did not affect the shapes of the double-reciprocal plots or the slopes of the Hill plots obtained with either the soluble or particulate enzymes (data not shown).

The actions of NaN_3, $NaNO_2$, and $C_6H_5NHNH_2$ on the guanylate cyclase activities of whole homogenates and 100 000*g* soluble and particulate fractions of renal cortex and outer and inner medulla were examined. Maximally effective concentrations of these agents were tested. As shown in Table II, responses to all three agents were greatest in cortical homogenates and much less prominent in medullary preparations. In cortex, maximal stimulation by both NaN_3 and $NaNO_2$ was twofold greater than that observed with $C_6H_5NHNH_2$. By contrast, $C_6H_5NHNH_2$ was the most effective agonist in outer medulla. In inner medulla, maximal actions of $C_6H_5NHNH_2$ and $NaNO_2$ were equivalent and exceeded those of azide. Although a 2-min lag period of enzyme activation was apparent with all three agonists in each tissue, preincubation of the agonists and tissue homogenates at 37 °C for periods of 5 to 10 min did not alter their relative effectiveness in the three tissues. Combination of 100 000*g* supernatants from renal cortex and inner medulla did yield more NaN_3-stimulated

Excess $MnCl_2$ is assumed to be that amount in addition to the concentration of substrate (MnGTP).

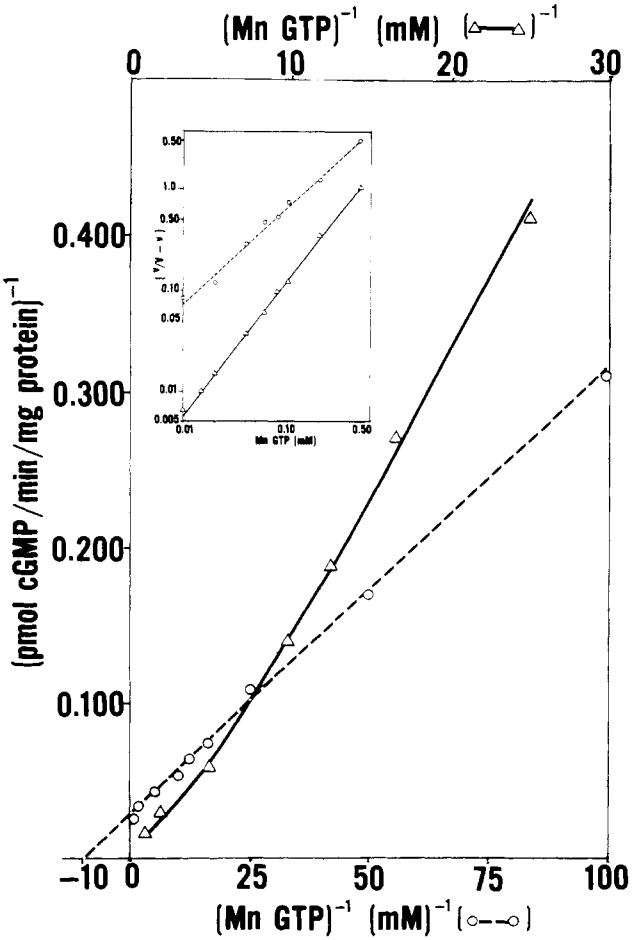

FIGURE 2: Double-reciprocal plot of initial velocity of guanylate cyclase activity vs. MnGTP concentration in the 100 000*g* soluble (○- - -○) and particulate (△—△) fractions of renal inner medulla. The concentration of MnGTP was varied, while the concentration of excess $MnCl_2$ was held constant at 3 mM. The insert shows the Hill plot of guanylate cyclase activity obtained using the 100 000*g* soluble (○- - -○) and particulate (△—△) fractions at various concentrations of MnGTP from 0.01 to 0.5 mM at a constant excess $MnCl_2$ concentration of 3 mM. The results shown are the means of duplicate determinations from the same experiment repeated twice.

TABLE III: The Effect of $NaNO_2$ on the Divalent Cation Requirement of Guanylate Cyclase in the 100 000*g* Fractions of Renal Cortex and Inner Medulla.[a]

Tissue Fraction	Sole Divalent Cation in Assay					
	4 mM $MnCl_2$		4 mM $CaCl_2$		4 mM $MgCl_2$	
	—	$NaNO_2$	—	$NaNO_2$	—	$NaNO_2$
Cortex 100 000*g* supernatant	155 ±8	3565[b] ±150	12[c] ±1	15[c] ±1	16[c] ±2	1823[b,c] ±90
Inner medulla 100 000*g* supernatant	48 ±2	192[b] ±8	3.8[c] ±0.2	4.3[c] ±0.2	7.2[c] ±0.3	92[b,c] ±5
Inner medulla 100 000*g* pellet	82 ±4	73 ±3	15[c] ±0.8	13[c] ±0.6	14[c] ±0.5	15[c] ±0.8

[a] Guanylate cyclase activities of the 100 000*g* soluble fractions of cortex and inner medulla and the 100 000*g* particulate fraction of inner medulla were determined in the presence and absence of 10 mM $NaNO_2$, with 4 mM $MnCl_2$, $CaCl_2$, or $MgCl_2$ as sole metal cofactor. Reaction mixtures were otherwise the same as described in Materials and Methods. Results are expressed as pmol min^{-1} (mg protein)$^{-1}$ in the fraction studied. Each value represents the mean ± SEM of duplicate assays pooled from three separate experiments. [b] Indicates p at least < 0.005 compared with corresponding control in the absence of $NaNO_2$. [c] Indicates p at least < 0.005 compared with corresponding value with 4 mM $MnCl_2$

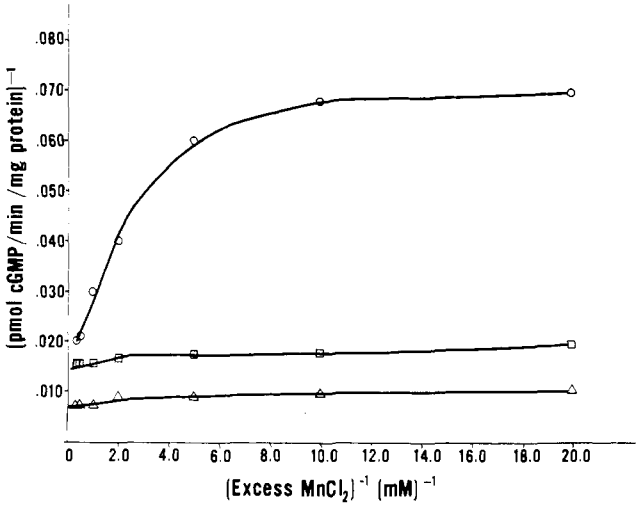

FIGURE 3: Double-reciprocal plot of initial velocity of guanylate cyclase activity vs. excess $MnCl_2$ concentration examined at a constant substrate concentration (1 mM MnGTP). The data shown were obtained with the 100 000*g* soluble fraction of inner medulla in the presence (Δ—Δ) and absence of 10 mM $NaNO_2$ (O—O) and with the 100 000*g* particulate fraction of this tissue in the absence of 10 mM $NaNO_2$ (□—□) The results shown are the means of duplicate determinations from the same experiment repeated twice.

activity than was present in the supernatants assayed separately. Thus, NaN_3-stimulated activity in the 100 000*g* supernatant from renal cortex was 246, while that from inner medulla was 6 pmol per min per assay. Combination of the two fractions yielded 358 rather than the expected 252 pmol per min per assay. Of the three agents tested, only NaN_3 was effective in increasing guanylate cyclase activity in a particulate fraction, and this effect was confined to renal cortex. In cortex, the increase in particulate activity with NaN_3 (5-fold) was much smaller than that observed with soluble activity (20-fold). As shown in Table II, $NaNO_2$ and $C_6H_5NHNH_2$ had no significant effect on enzyme activity in the particulate fractions of any of the tissues, but increased the activities of the soluble fractions of renal cortex and inner and outer medulla (2–23 times). The latter agents were without effect on particulate activity when examined over a wide range of concentrations (0.1–20 mM) in the guanylate cyclase reaction mixture and responsiveness of the particulate fractions was not expressed by preincubation with these agonists. The Triton solubilized particulate enzyme of inner medulla was also unresponsive to NaN_3, $NaNO_2$, and $C_6H_5NHNH_2$ (not shown). Recoveries of basal, NaN_3, $NaNO_2$, and $C_6H_5NHNH_2$ stimulated activities in the 100 000*g* soluble and particulate fractions were 85–95% of whole homogenate activity (Table II).

Table III shows the effect of $NaNO_2$ on the divalent cation requirements of guanylate cyclase in the 100 000*g* soluble fraction of renal cortex and the 100 000*g* soluble and particulate fractions of renal inner medulla. In the absence of $NaNO_2$, 4 mM $CaCl_2$ and 4 mM $MgCl_2$ were not effective substitutes for 4 mM $MnCl_2$ as sole metal cofactor with any of the 100 000*g* tissue fractions studied. Thus, guanylate cyclase activity measured in the presence of 4 mM $MgCl_2$ or $CaCl_2$ was only 10 to 15% of that observed with 4 mM $MnCl_2$ in the reaction mixture. By contrast, the guanylate cyclase activity of the 100 000*g* supernatant fractions of renal cortex or inner medulla measured in the presence of 10 mM $NaNO_2$ and with 4 mM $MgCl_2$ as the sole metal cofactor was approximately 50% of that observed with 4 mM $MnCl_2$ Indeed with Mg^{2+}, responses to $NaNO_2$ of the soluble enzymes from both cortex and inner medulla, expressed as fold-increase over basal, were markedly enhanced. Mg^{2+} similarly enhances the responsiveness of the cortical soluble enzyme to NaN_3, as previously reported (DeRubertis and Craven, 1976b). $CaCl_2$ could not effectively substitute for $MnCl_2$ or $MgCl_2$ in expressing $NaNO_2$ stimulated activity under these conditions. As is also shown in Table III, $NaNO_2$ had no effect on the divalent metal ion requirement of the 100 000*g* particulate fraction of renal inner medulla. Guanylate cyclase activity in the latter fraction, in the presence or absence of $NaNO_2$ and with either $CaCl_2$ or $MgCl_2$ as sole metal cofactor, was only 10 to 15% of the activity in the presence of $MnCl_2$.

The effect of $NaNO_2$ on guanylate cyclase activity determined at varying excess $MnCl_2$ concentrations (0–3 mM)[2] was assessed in the 100 000*g* soluble and particulate fractions of renal inner medulla (Figure 3) in the absence of Triton. The concentration of MnGTP was held constant at 1 mM. Double-reciprocal plots of the data obtained with the 100 000*g* soluble fraction in the absence of $NaNO_2$ are nearly linear

from 0.5 to 3 mM excess $MnCl_2$ but show negative cooperativity below 0.5 mM. By contrast, double-reciprocal plots of initial velocity vs. excess $MnCl_2$ concentration with the particulate enzyme in the absence of $NaNO_2$ have a near-zero slope at saturating MnGTP (1 mM). Addition of 10 mM $NaNO_2$ to the reaction mixture reduced the excess Mn^{2+} requirements of the soluble enzyme. Thus, maximal activity of the soluble enzyme in the absence of $NaNO_2$ was observed when the ratio of total $MnCl_2$/GTP was 4, whereas, in the presence of $NaNO_2$, this ratio was reduced to 2. Furthermore, the shape of double-reciprocal plots of initial velocity vs. excess $MnCl_2$ concentration for the soluble enzyme in the presence of $NaNO_2$ resembled that for the particulate enzyme in the absence of $NaNO_2$ and had a near-zero slope. The effects of $NaNO_2$ on the excess Mn^{2+} kinetics of the soluble enzyme from renal cortex were similar to those described for the soluble enzyme in inner medulla and are not shown. The addition of $NaNO_2$ to reaction mixtures did not affect the shape of the curve obtained with the enzyme in the particulate fraction from renal inner medulla. The effects of $NaNO_2$ on the excess Mn^{2+} kinetics of the particulate enzyme were also examined at 0.25 mM MnGTP. At this latter substrate concentration, the slope of a double-reciprocal plot of initial velocity vs. excess $MnCl_2$ concentration with the particulate enzyme is greater than zero. However, under these conditions, $NaNO_2$ still failed to alter particulate guanylate cyclase activity.

Discussion

The present study illustrates clear differences in the subcellular distribution of guanylate cyclase in three regions of the kidney. The predominantly soluble localization of the enzyme in renal cortex is consistent with our own previous data (DeRubertis and Craven, 1976b) and those of others (Bohme, 1970; Kimura et al., 1975a; Criss et al., 1976). However, its predominantly particulate distribution in renal inner medulla is at variance with the results reported by Neer and Sukiennik (1975). In the latter study, the dissection techniques employed to minimize cortical contamination of the relatively small mass of medullary tissue were not reported, and apparently outer and inner medulla were not separated. The differences in the subcellular distribution of guanylate cyclase which we have observed in carefully separated areas of the rat kidney are consistent with recent findings in sheep renal medulla, where a predominance of particulate guanylate cyclase was also noted (Frey et al., 1976). Our results in the rat are most consistent with the possibility that distinct enzyme systems exist in soluble and particulate cell fractions of the kidney. These two systems can be differentiated on the basis of several properties. First, the activity of the soluble enzyme from all three tissues is more responsive to NaN_3, $NaNO_2$, and $C_6H_5NHNH_2$ than the particulate enzyme. Second, the soluble enzyme is activated by Ca^{2+}, whereas the particulate enzyme is inhibited by this cation. Third, the soluble enzyme is more dependent on the concentration of excess Mn^{2+} than the particulate enzyme. Finally, the soluble enzyme exhibits typical Michaelis–Menten kinetics with respect to its substrate (MnGTP), whereas the particulate enzyme displays apparent positive cooperative behavior. Accordingly, particulate guanylate cyclase could have an important regulatory function in the renal inner medulla, where it predominates. Many of the properties of the particulate enzyme from inner medulla reported here, such as lower Mn^{2+} requirements, have also been found to be characteristic of particulate guanylate cyclase from other tissues (Kimura and Murad, 1974, 1975a,b; Garbers et al., 1975; Chrisman et al., 1975; Gray and Drummond, 1976; Gray et al., 1976). However, the unresponsiveness of the particulate enzyme from renal medulla to several recently identified agonists of guanylate cyclase (DeRubertis and Craven, 1976b; Kimura et al., 1975b) has not previously been described.

The differences in the excess Mn^{2+} requirement of the soluble and particulate enzyme noted in inner medulla and in other tissues may have physiologic implications (Kimura and Murad, 1975a). The concentration of Mn^{2+} ion in the cell probably does not exceed 0.5 mM (Thiers and Vallee, 1957). Thus, under conditions which pertain in the intact cell, the activity of the soluble enzyme could be very low (Kimura and Murad, 1975a), and the predominance of the particulate enzyme in inner medulla may be an important determinant of the higher steady-state cGMP levels in this region of the kidney. Both the reduced requirement for Mn^{2+} and the unique kinetic properties of the particulate enzyme are consistent with this possibility. A similar correlation between high basal cGMP levels and the proportion of total guanylate cyclase activity in the particulate form exists in some hepatomas and renal cortical tumors (Kimura and Murad, 1975c; Criss et al., 1976; DeRubertis and Craven, 1976c). Our data in kidney also imply that one of the effects of $NaNO_2$ is to reduce the excess Mn^{2+} requirement of the soluble enzyme and to allow expression of stimulated enzyme activity in the presence of Mg^{2+} as the sole divalent cation Indeed, with Mg^{2+} the relative responsiveness to $NaNO_2$ of the soluble enzymes from both the cortex and inner medulla exceeds that observed with Mn^{2+}, although absolute $NaNO_2$-stimulated activity is higher with Mn^{2+} (Table III). As previously reported (DeRubertis and Craven, 1976b), the effects of NaN_3 on the metal requirement of soluble guanylate cyclase from cortex are analogous to those described here for $NaNO_2$, and both of these agonists increase cGMP levels in renal cortical (DeRubertis and Craven, 1976b, 1976d) and medullary slices (DeRubertis and Craven, manuscript in preparation). Thus, it seems possible that the effects of $NaNO_2$ and related agonists on the metal requirements of the soluble enzyme could be linked to their ability to enhance cGMP under conditions of low Mn^{2+} availability which pertain in intact cells.

Finally, significant differences were evident in the responses of soluble guanylate cyclase from renal cortex and outer and inner medulla to the agonists tested (Table II). The responses of soluble activity to NaN_3, $NaNO_2$, and $C_6H_5NHNH_2$ were clearly greatest in cortex. However, stimulation of soluble enzyme activity from outer and inner medulla by these same agonists could also be distinguished (Table II). Thus, in addition to differences in subcellular distribution, these results imply that the regulation and properties of soluble enzyme activity of cortex and outer and inner medulla are also distinct. The data of Mittal and co-workers suggest that the unresponsiveness of soluble guanylate cyclase activity from brain to azide may be due to the absence of a macromolecular factor required for expression of azide activity and present in liver (Mittal et al., 1975). Analogous to the results they observed with combined soluble fractions from brain and liver, combination of soluble fractions of renal cortex and inner medulla resulted in expression of more NaN_3 responsive activity than was predicted by assay of these two fractions separately. Accordingly, the relatively poor response of soluble guanylate cyclase from renal medulla to azide and related agonists may be due to lack of the macromolecular factor implicated by the studies of Mittal et al. (1975). Whatever the ultimate mechanisms involved, the distinctive features of the soluble guanylate cyclase system in each region of the kidney, as well as the differences in subcellular enzyme distribution, could both

subserve the specialized metabolic and excretory functions of these three renal tissues.

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