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Biosynthesis of Guanidinoacetic Acid in Isolated Renal Tubules

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Summary: Guanidinoacetic acid, a precursor of creatine, is an essential substrate for muscle energy metabolism. Since guanidinoacetic acid has been reported to be synthesized from arginine and glycine by glycine amidinotransferase (transamidinase) in kidney homogenates or slices, the purpose of this study was to provide evidence of guanidinoacetic acid synthesis in isolated tubules from rat kidneys, and to clarify the mechanism regulating it. Isolated rat tubules were incubated with various substrates. Guanidinoacetic acid was separated by high performance liquid chromatography and measured fluorometrically.

Results obtained were as follows:

(1) Guanidinoacetic acid was synthesized from arginine or canavanine and glycine in isolated rat tubules.

(2) *D,L*-Norvaline, ornithine and methionine suppressed guanidinoacetic acid synthesis.

(3) Creatine suppressed guanidinoacetic acid synthesis, i.e. creatine was a negative feedback inhibitor of guanidinoacetic acid synthesis in this *in vitro* system.

(4) Guanidinoacetic acid was not synthesized from hydroxyurea, citrulline, argininosuccinic acid or canaline.

These data demonstrate that guanidinoacetic acid is synthesized only from arginine or canavanine and glycine, and that the guanidine cycle may not function fully in the rat renal tubule.

Introduction

Guanidinoacetic acid is synthesized by glycine amidinotransferase[1]) (transamidinase) mainly in the kidney (1). Part of the guanidinoacetic acid synthesized in the kidney is transported to the liver, where it is methylated to creatine (2) and the rest is excreted in the urine (3).

Guanidinoacetic acid itself has been reported to have certain physiological roles, e.g., the stimulation of insulin secretion (4). However, the biological significance of guanidinoacetic acid in the body is most apparent when guanidinoacetic acid synthesis is decreased in the kidney. In chronic renal failure, the decrease of guanidinoacetic acid synthesis in the kidney results in a decreased creatine content in various organs, including skeletal muscle (5). On the other hand, guanidinoacetic acid excretion in the urine is closely correlated with creatinine clearance (6). Urinary excretion of guanidinoacetic acid, which may reflect a metabolic function of the kidney, is decreased when renal function deteriorates (3), and the measurement of urinary guanidinoacetic acid excretion has been reported to be an effective index for the detection of renal dysfunctions such as gentamicin nephropathy (7), hypertensive renal disease (8) and rejection of renal transplantation (9).

[1]) Glycine-amidinotransferase (EC 2.1.4.1)
Ornithine transcarbamylase (EC 2.1.3.3)
Argininosuccinic acid synthetase (EC 6.3.4.5)
Argininosuccinic acid lyase (EC 4.3.2.1)
Guanidinoacetate methyltransferase (EC 2.1.1.2)
Carbamylphosphate synthetase (EC 6.3.4.16)
Arginase (EC 3.5.3.1)

Arginine or canavanine plus glycine serve as substrates for guanidinoacetic acid synthesis in rat kidney homogenates (10–15) and slices (1). Transamidinase[1]) activity in the kidney is regulated by hormones (16) or dietary factors (16, 17) *in vivo*. Creatine feeding was reported to suppress transamidinase activity in the kidney (16, 17).

Ungar et al. (18) and *Fitch* et al. (19) added growth hormone and creatine, respectively, to a guanidinoacetic acid synthesizing system in rat kidney homogenates, but could not reproduce the *in vivo* phenomenon. Furthermore, only one study has demonstrated the regulatory mechanism of guanidinoacetic acid synthesis *in vitro*, in which ornithine suppressed guanidinoacetic acid synthesis in rat kidney homogenates (10).

The purpose of this study was to investigate the mechanism of guanidinoacetic acid synthesis *in vitro* by using various substrates in isolated renal tubules from rat kidneys. We propose a possible pathway for the regulation of guanidinoacetic acid synthesis.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, Tokyo, Japan) weighing 200 to 250 g were used in these experiments.

Biochemical materials

Collagenase (type I), bovine serum albumin (fraction V), canavanine, canaline and argininosuccinic acid were purchased from Sigma Chemical (St. Louis, MO). Arginine, glycine, *D,L*-norvaline, ornithine and creatine were purchased from Wako Pure Chemical (Osaka, Japan). Hydroxyurea and citrulline were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were of analytical grade and purchased from commercial sources.

Isolation procedure for renal tubules

Rat renal tubules were isolated by the method of *Obara* et al. (20). In brief, rats were anaesthetized by the intraperitoneal administration of 50 mg/kg body weight phenobarbital. The left kindney was perfused *in situ* through the renal artery with ice-cold *Hanks'* solution (1 mmol/l $CaCl_2$) containing 1 g/l collagenase (Sigma type I) and 1 g/l bovine serum albumin. Kidney slices, 1 mm in thickness, were prepared and incubated at 37 °C for 30 min in the same solution bubbled with 95% oxygen gas. After incubation, the slices were washed with ice-cold *Hanks'* solution (0.25 mmol/l $CaCl_2$). The mixture was then stirred with a vortex mixer for 15 s, and the upper turbid medium was transferred to a 50 ml centrifuge tube (Corning Laboratory Science Co., NY). This mechanical dispersion procedure was repeated three times. The particles of dissociated tissue were removed by filtration through a 1 mm nylon mesh. After allowing the suspension containing tubules and cells to stand for 5 min in ice, the upper fraction containing almost all cells was carefully pipetted out. The precipitate was resuspended in *Hanks'* solution and centrifuged at 300 *g* for 5 min. The pellet was resuspended in *Hanks'* solution and used as a tubular suspension.

Assay of guanidinoacetic acid synthesized in isolated renal tubules

The isolated tubules were incubated in 1 ml *Hanks'* solution containing various substrates with shaking at 60 min^{-1} in a 1.5 ml microtube (Sarstedt, Germany) at 37 °C. At the end of each incubation, the tissue was disintegrated by pulsatile sonication for 30 s with a Sonifier (Branson Sonic Power Co., Conn. USA), followed by the addition of 89 µl of 1 kg/l trichloroacetic acid. The trichloroacetic acid extracts were filtered through a 0.45 µm Millipore filter (Millipore Limited Japan). The pH of the aliquots was adjusted to 2.2 with 2 mol/l NaOH. Aliquots of the adjusted extracts were used for guanidinoacetic acid determination.

Guanidinoacetic acid was separated by high performance liquid chromatography (HPLC) using a Guanidinopack II (Japan Spectroscopic Co., Tokyo, Japan), eluted with 0.2 mol/l sodium citrate buffer, pH 3.0, at a flow rate of 0.5 ml/min for 10 min, followed by 0.2 mol/l sodium citrate buffer, pH 3.5, at 0.5 ml/min for 5 min, and determined fluorometrically using 9,10-phenanthrenequinone for the post-labelling method (21). Fluoroscence was measured (excitation at 365 nm, emission at 495 nm) with a fluorescence detector (Model FP 110-C, Japan Spectroscopic Co., Tokyo, Japan). The areas of chromatographic peaks were determined with a Chromatocorder 12 (System Instrument Co., Tokyo, Japan) with a baseline display connected between the fluoromonitor and the recorder. The tissue protein concentration was determined by the method of *Lowry* et al. (22).

Calculation and statistics

The ratio of the area of each sample in HPLC to the standard area was calculated. Amounts of guanidinoacetic acid are shown as µmol/g tissue protein. All results are expressed as mean ± SE. Statistical analyses were performed by the *Mann-Whitney* rank sum test (23).

Results

Guanidinoacetic acid synthesis in isolated renal tubules

We incubated the isolated renal tubules with various concentrations of arginine or canavanine and 1 mmol/l glycine. As shown in figure 1, the dose-response relationship between guanidinoacetic acid synthesis and arginine was observed in the range of 0 to 2.0 mmol/l of arginine. Guanidinoacetic acid synthesis from canavanine was saturated by less than 2 mmol/l of canavanine (data not shown). The isolated renal tubules were also incubated with various concentrations of glycine and 1 mmol/l arginine. The dose-response relationship between glycine and guanidinoacetic acid synthesis was observed in the range from 0 to 2.0 mmol/l of glycine (data not shown). Furthermore, we incubated the isolated renal tubules with 1 mmol/l arginine or 1 mmol/l canavanine and 1 mmol/l glycine for 0–90 min. As shown in figure 2, guanidinoacetic acid synthesis increased up to 90 min, depending on the incubation time.

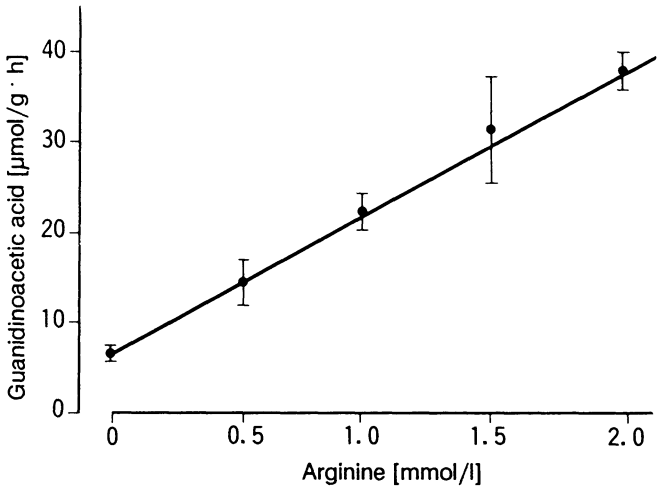

Fig. 1. Dose-response curve of arginine concentration versus guanidinoacetic acid synthesis in isolated renal tubules. The isolated tubules were incubated for 60 min with various concentrations of arginine and 1 mmol/l glycine. Values are means ± SE of 4 samples (r = 0.999).

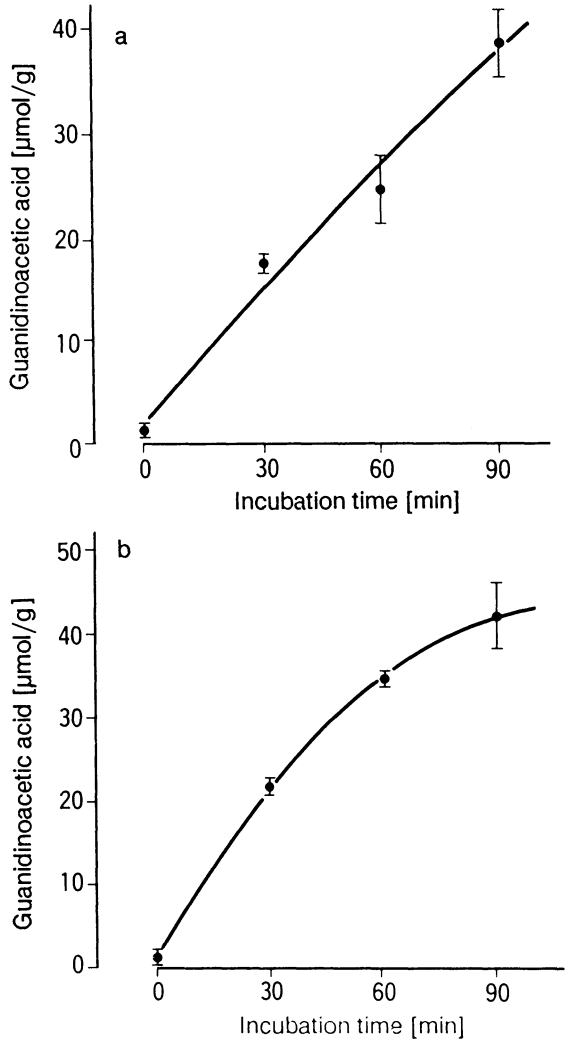

Fig. 2. Time course of guanidinoacetic acid synthesis in isolated rat tubules. The isolated tubules were incubated with 1 mmol/l arginine (a) or 1 mmol/l canavanine (b) and 1 mmol/l glycine for 30, 60, or 90 min. Values are means ± SE of 4 samples (r = 0.985, 0.999, respectively).

Regulation of guanidinoacetic acid synthesis by various substrates in the isolated renal tubules

D,L-Norvaline and ornithine have been reported to suppress transamidinase activity in human pancreas homogenates (24) and isolated rat hepatocytes (25). Methionine also suppresses guanidinoacetic acid synthesis in isolated rat hepatocytes (25). However, there has been only one report concerning the regulation of the guanidinoacetic acid synthesis in the kidney; *Ratner* et al. (10) reported that ornithine suppressed

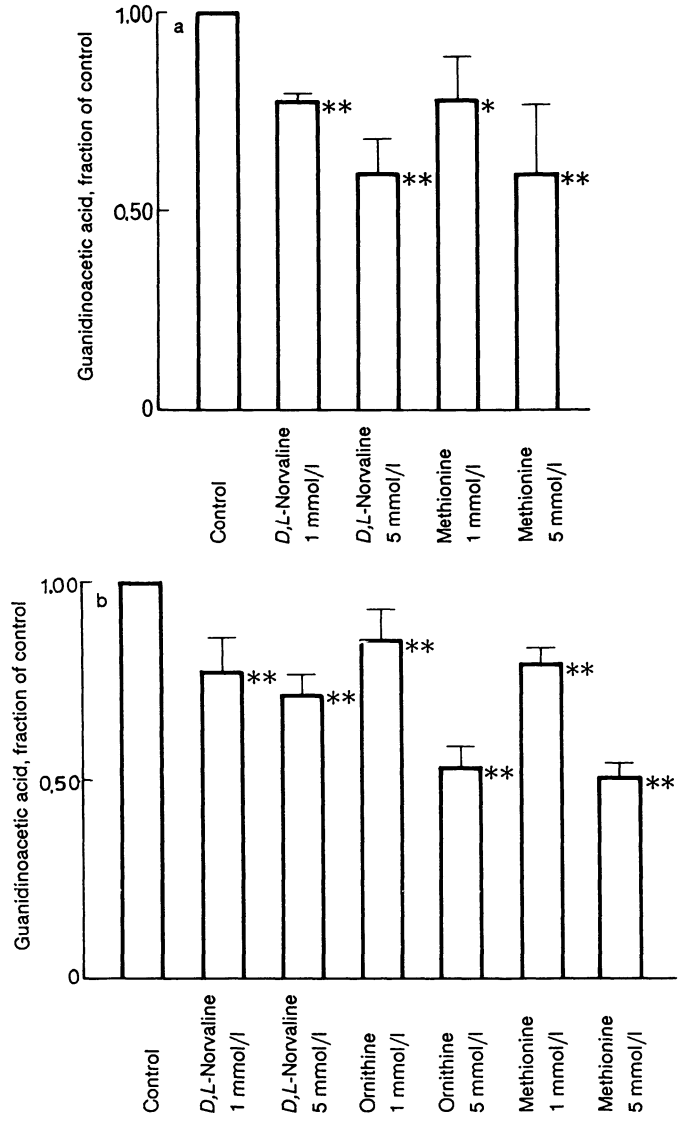

Fig. 3. Regulation of guanidinoacetic acid synthesis by various substrates in isolated rat tubules.
(a) The isolated tubules were incubated with 1 mmol/l arginine and 1 mmol/l glycine with or without 1 or 5 mmol/l *D,L*-norvaline and methionine for 60 min.
(b) 1 or 5 mmol/l *D,L*-norvaline, ornithine and methionine were added to the isolated renal tubules incubated with 1 mmol/l canavanine and 1 mmol/l glycine for 60 min.
Values are means ± SE of 5 samples.
* $P < 0.05$, ** $P < 0.01$.

guanidinoacetic acid synthesis from arginine and glycine. The isolated renal tubules were incubated with 1 mmol/l arginine or 1 mmol/l canavanine and 1 mmol/l glycine with or without the addition of 1 or 5 mmol/l *D,L*-norvaline, ornithine or methionine. As shown in figure 3a, 1 or 5 mmol/l *D,L*-norvaline and methionine significantly suppressed guanidinoacetic acid synthesis from arginine and glycine as substrates. Figure 3b shows that 1 or 5 mmol/l *D,L*-norvaline, ornithine and methionine significantly inhibited guanidinoacetic acid synthesis from canavanine and glycine.

Effect of various concentrations of creatine on guanidinoacetic acid synthesis in the isolated renal tubules

We added various concentrations of creatine to the isolated renal tubules incubated with arginine or canavanine and glycine. The addition of 100 and 300 mg/l of creatine suppressed guanidinoacetic acid synthesis from arginine and glycine as substrates (fig. 4).

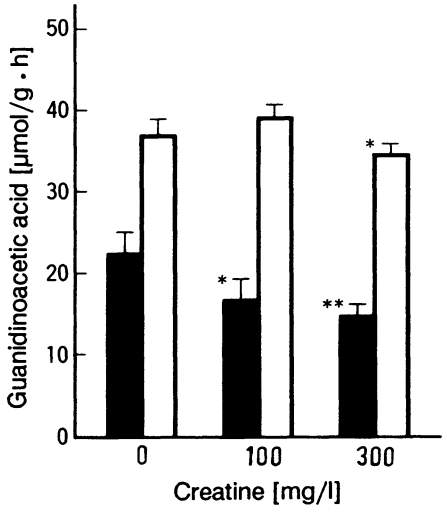

Fig. 4. Effect of creatine on guanidinoacetic acid synthesis in isolated rat tubules. Various concentrations of creatine were added to isolated tubules incubated with 1 mmol/l arginine + 1 mmol/l glycine (■) or 1 mmol/l canavanine + 1 mmol/l glycine (□) for 60 min. Values are means ± SE of 5 samples.
* $P < 0.05$, ** $P < 0.01$.

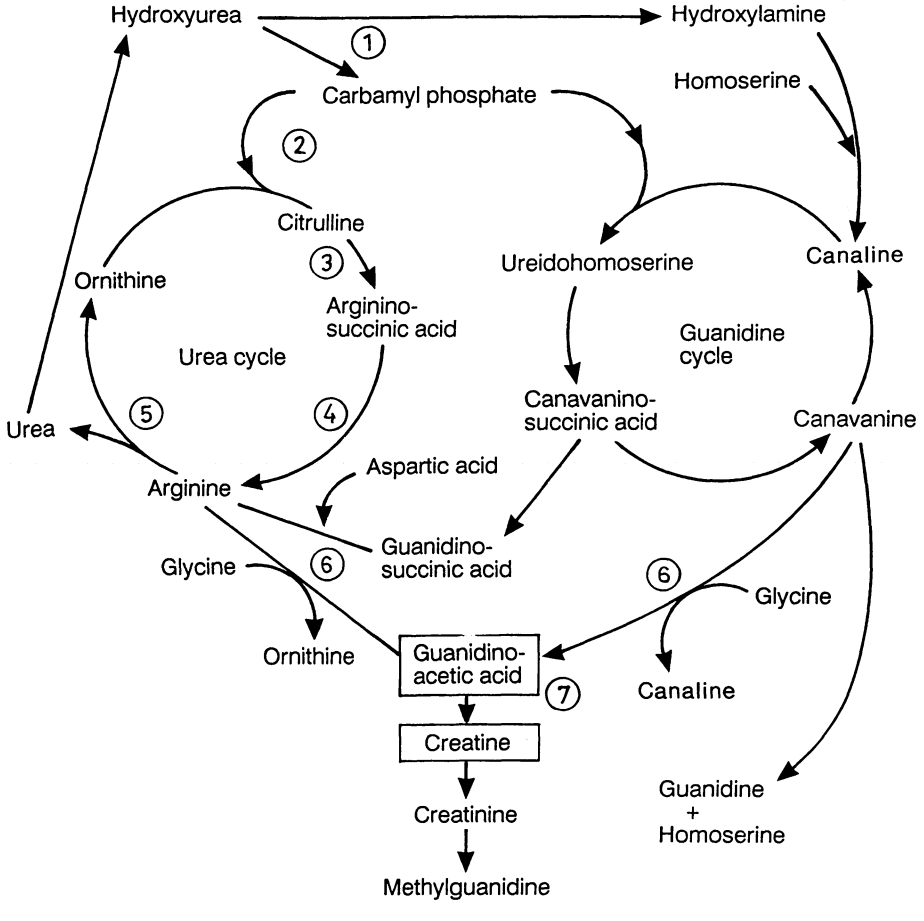

Fig. 5. Metabolic reactions of the guanidine and urea cycles.
Enzymes
① Carbamylphosphate synthetase
② Ornithine transcarbamylase
③ Argininosuccinic acid synthetase
④ Argininosuccinic acid lyase
⑤ Arginase
⑥ Glycine-amidinotransferase (transamidinase)
⑦ Guanidinoacetate methyltransferase

When canavanine and glycine were used as substrates, 100 and 300 mg/l of creatine also significantly suppressed guanidinoacetic acid synthesis. These results suggest the negative feedback control of guanidinoacetic acid synthesis by creatine *in vitro*.

Possible metabolic pathway of guanidinoacetic acid synthesis in the isolated renal tubules

Among the urea cycle enzymes shown in figure 5, ornithine transcarbamylase[1]), argininosuccinic acid synthetase and argininosuccinic acid lyase[1]) have been reported to exist in the renal medulla at low levels (26, 27). On the other hand, there has been no evidence as to whether the guanidine cycle exists in the kidney, while it is considered to exist in the liver (28). We tried to determine the presence of enzymes of the urea and guanidine cycles in isolated renal tubules by determining guanidinoacetic acid synthesis. We incubated the isolated renal tubules with 10 mmol/l glycine and 1 mmol/l of either arginine, canavanine, canaline, hydroxyurea, citrulline or argininosuccinic acid.

As shown in figure 6, guanidinoacetic acid was clearly synthesized from arginine and canavanine, but there was no synthesis from hydroxyurea, citrulline, agrininosuccinic acid or canaline.

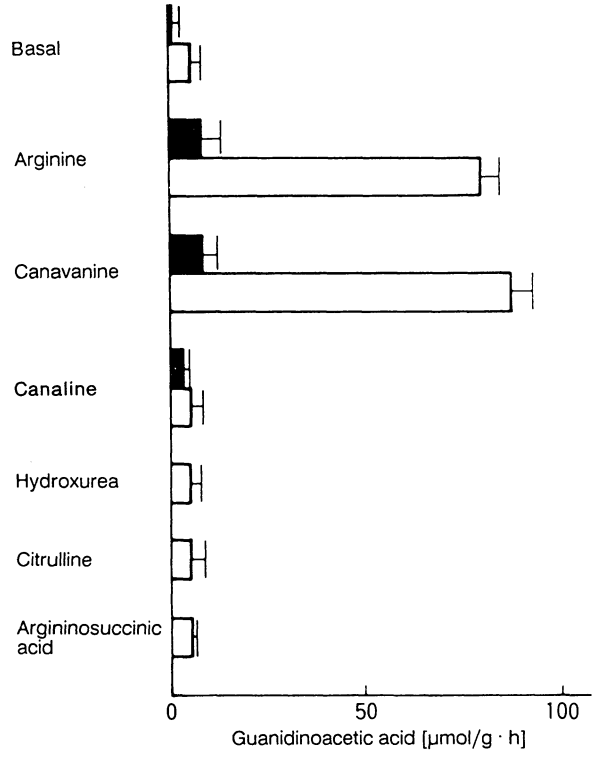

Fig. 6. Guanidinoacetic acid synthesis in isolated rat tubules from various substrates. The isolated tubules were incubated without (■) and with 10 mmol/l glycine (□) and 1 mmol/l of various substrates indicated on the ordinate for 60 min. Values are means ± SE of 4 samples.

Discussion

Creatine is synthesized according to the following two steps.

$$\text{Arginine} + \text{Glycine} \rightleftharpoons \text{Guanidinoacetic acid} + \text{Ornithine} \quad [A]$$

$$\text{Guanidinoacetic acid} + \text{S-Adenosylmethionine} \rightarrow \text{Creatine} + \text{S-Adenosylhomocysteine} \quad [B]$$

Transamidinase[1]) and guanidinoacetate-methyltransferase[1]) catalyse reactions [A] and [B], respectively (1, 2). Arginine in reaction [A] has been reported to be synthesized from citrulline mainly in the kidney (29, 30). Reaction [A] also occurs mainly in the kidney (15).

Guanidinoacetic acid synthesis in the kidney has been investigated with rat kidney homogenates (10–15) and slices (1, 16). Although the numerous previous studies on guanidinoacetic acid synthesis used kidney homogenates (10–15), the present studies were performed on isolated renal tubules consisting mainly of proximal convoluted tubule, where transamidinase was observed by an immunofluorescence technique (31). Furthermore, the specificity and sensitivity of our HPLC and fluorometric methods (21) permit the determination of lower levels of guanidinoacetic acid synthesis from low substrate concentrations, compared with previous studies (1, 10–15). Thus, it is likely that the guanidinoacetic acid synthesis measured in the present study reflects a more physiological metabolism.

Transamidinase activity has been shown to be regulated by dietary (16, 17) or hormonal factors (17), *in vivo*. However, the regulation of guanidinoacetic acid synthesis by various substrates has never been fully investigated in renal tubules. In this study, we demonstrated the effect of various substrates on the regulation of guanidinoacetic acid synthesis in isolated renal tubules. *D,L*-Norvaline, a multiple enzyme inhibitor of the urea cycle (32), inhibited guanidinoacetic acid synthesis in isolated renal tubules, This finding is consistent with the data in isolated hepatocytes (25) and human pancreas homogenates incubated with canavanine and glycine as substrates (24). When arginine and glycine were used as substrates, however, our results were not consistent with the data in isolated rat hepatocytes, in which *D,L*-norvaline did not inhibit guanidinoacetic acid synthesis (25). This study may provide the first evidence of the suppression of transamidinase activity by *D,L*-norvaline. Ornithine, a member of urea cycle that promotes urea synthesis (33), has been reported to suppress guanidinoacetic

acid synthesis from arginine and glycine in rat kidney homogenates (10). This may be a simple negative feedback control of guanidinoacetic acid synthesis. We found that ornithine inhibited transamidinase activity by using canavanine and glycine as substrates. Furthermore, we found that methionine also suppressed guanidinoacetic acid synthesis in isolated renal tubules. It is highly likely that guanidinoacetic acid synthesized from arginine or canavanine and glycine is methylated to creatine. However, as reported previously, we failed to detect creatine synthesis in microdissected proximal convoluted tubules, isolated glomeruli or kidney homogenates with guanidinoacetic acid or arginine and glycine plus methionine (34). At present, we postulate that methionine may have an inhibitory effect on transamidinase activity in the kidney.

It has been reported that creatine feeding suppresses transamidinase activity in rat kidney homogenates (16). Transamidinase is considered to be a rate limiting enzyme for creatine synthesis. In contrast, *Fitch* et al. (20) have reported that the addition of creatine did not inhibit transamidinase activity in rat kidney homogenates. In our study, the addition of 300 mg/l creatine, which is about 1/10 of the concentration used in *Fitch*'s study, suppressed guanidinoacetic acid synthesis when either arginine or canavanine were present as the amidine donor. The mechanism of the decrease of transamidinase activity by creatine feeding is of considerable interest. *McGuire* et al. (35) reported that the relative rate of synthesis and the mRNA levels of transamidinase were reduced in creatine-fed rats. Thus, creatine was thought to inhibit transamidinase activity by affecting its rate of synthesis at a pretranslational stage. It is unlikely that creatine could be a signal for mRNA functional activities in kidney homogenates (35), in which the cell structures are destroyed. In contrast, in our isolated renal tubules, where cell structures were preserved, the addition of creatine may reduce transamidinase activity at a pretranslational stage.

Among the urea cycle enzymes in the kidney, ornithine transcarbamylase, argininosuccinic acid synthetase and argininosuccinic acid lyase are present in the medulla in very small amounts (26, 27), and the distribution of carbamylphosphate synthetase is reported to coincide with ornithine transcarbamylase activity (27). The fact that guanidinoacetic acid could not be produced from hydroxyurea may confirm the data that only very small amounts of carbamylphosphate synthetase are present (27). Since our isolated tubules consisted mainly of cortical proximal tubules and since argininosuccinic acid synthetase is located mainly in the medulla, as stated above (26), it seems reasonable that citrulline plus glycine did not produce guanidinoacetic acid. In contrast, *Funahashi* et al. (29) and *Featherston* et al. (30) have reported that the kidney synthesizes a large portion of endogenous arginine from citrulline *in vivo*. In these studies, citrulline was converted by argininosuccinic acid synthetase and argininosuccinic acid lyase in the renal medulla. Recently, *Saheki* et al. (36) used the immunofluorescence technique to show that argininosuccinic acid synthetase is localized in the cell structures of proximal convoluted tubules. Even if argininosuccinic acid synthetase is located in the proximal convoluted tubules, guanidinoacetic acid synthesis from citrulline might not be recognized because argininosuccinic acid lyase is localized mainly in the medulla (26). In our studies, guanidinoacetic acid synthesis from argininosuccinic acid and glycine was not observed in renal tubules. We confirmed existing information on the presence of urea cycle enzymes in the kidney by determining guanidinoacetic acid synthesis in isolated rat tubules. The guanidine cycle has been proposed to exist in the liver and to interrelate with the urea cycle (28). Canaline, which is part of the guanidine cycle (28), did not produce guanidinoacetic acid in isolated rat tubules in our studies. From these results, it is highly likely that the entire guanidine cycle or certain enzymes in this cycle are depleted in the rat kidney.

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