Effects of dietary P_i on the renal Na^+ -dependent P_i transporter NaPi-2 in thyroparathyroidectomized rats

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Dietary P_i and parathyroid hormone (PTH) are two most important physiological and pathophysiological regulators of P_i re-absorption in the renal proximal tubule. Effects of dietary P_i on Na^+ / P_i co-transporter NaPi-2 were investigated in thyroparathyroidectomized (TPTX) rats. NaPi-2 protein and mRNA in the kidney cortex of TPTX rats were increased $\approx$ 3.8- and 2.4-fold in amount respectively compared with those in the sham-operated animals. Administration of PTH to the TPTX rats resulted in a decrease in the amount of NaPi-2 protein, but not in the abundance of NaPi-2 mRNA. Deprivation of dietary P_i in the TPTX rats did not affect the amount of NaPi-2 mRNA

and protein. In the P_i -deprived TPTX rats, feeding of a high- P_i diet resulted in marked decreases in P_i transport activity and the amount of NaPi-2 protein in the superficial nephrons. Immunohistochemical analysis demonstrated that administration of PTH to TPTX rats resulted in a decrease in NaPi-2 immunoreactivity from both superficial and juxtamedullary nephrons within 4 h. Switching TPTX animals from a low- P_i diet to the high- P_i diet decreased NaPi-2 immunoreactivity from superficial nephrons, but not from juxtamedullary nephrons, within 4 h. These results suggest that dietary P_i could regulate the amount of NaPi-2 protein in the superficial nephrons in a PTH-independent manner.

INTRODUCTION

Re-absorption of inorganic phosphate (P_i) by the kidney occurs predominantly in the proximal tubule and begins with the movement of P_i across the luminal brush-border membrane (BBM) of proximal tubular cells [1–3]. This influx of P_i into the cells across the BBM is dependent on the transmembrane Na^+ gradient and is regulated by dietary P_i and parathyroid hormone (PTH) [4–6].

At least three types of Na^+ -dependent P_i (Na^+/P_i) co-transporters (types I–III) have been identified in the proximal tubules of rat kidney [3,7]. cDNA encoding the type II Na^+/P_i co-transport system (NaPi-2) was identified by expression cloning [8], and expression of this protein was shown to be regulated by dietary P_i and PTH [3,9,10]. When the food was changed from a low- P_i diet to a high- P_i diet, the Na^+/P_i transport activity was rapidly inhibited. This effect of dietary P_i is characterized by a decrease in the apparent maximal rate of transport (V_{max}) for P_i , with a decrease in the amount of NaPi-2 protein [9]. In acute phase, the effect of the high- P_i diet on P_i transport is independent of *de novo* protein synthesis and attributed to recycling of endocytosed plasma membrane [9,10].

Parathyroidectomy decreases renal P_i excretion, and, conversely, injection of PTH increases it [1,2]. Administration of PTH to rats *in vivo* specifically reduces the activity of Na^+/P_i transport in BBM vesicles (BBMVs) isolated from the kidney [5]. The inhibitory influence is also shown in cultured epithelial opossum kidney (OK) cells [6]. Binding of PTH to its receptor on the basolateral membrane of renal-tubular cells activates multiple intracellular signalling pathways which may induce endocytotic internalization of P_i transporters [1,2]. However, it is unclear whether two stimuli, dietary P_i and PTH, are additive or not.

We have now investigated the possibility that the effect of a high- P_i diet is mediated, at least in part, through PTH-induced endocytotic internalization of NaPi-2.

MATERIALS AND METHODS

Animals and diets

Intact and chronically thyroparathyroidectomized (TPTX) male Sprague–Dawley rats (body weight 178–230 g) were purchased from SLC (Shizuoka, Japan). Urinary excretion of P_i and calcium of all rats was determined using metabolic cages. Successful thyroparathyroidectomy was indicated by a substantial decrease in P_i excretion and an increase in calcium excretion compared with intact rats [11]. The TPTX rats were maintained on laboratory chows for 35 days before experiments. An effect of PTH on the regulation of renal Na^+/P_i co-transport in TPTX rats was investigated after two intravenous injections of bovine PTH (amino acids 1–34; Sigma) at a dose of 7.5 μ g/100 g of body weight per injection.

To investigate an effect of dietary P_i on the regulation of Na^+/P_i co-transporter in the TPTX rats, they were maintained in plastic cages and fed on a low- P_i diet containing 0.6% calcium, 0.02% phosphorus and vitamin D_3 (4.4 i.u./g) between 09:00 and 11:00 h for 14 days [9]. Sham-operated animals received a control diet containing 0.6% calcium and 0.6% phosphorus. On day 15 the TPTX rats were fed a diet containing a high percentage (1.2%) of phosphorus, and, at various times after this final feeding, their kidneys were rapidly removed under pentobarbital anaesthesia. One half of each kidney was used for RNA isolation, the other half for isolation of BBMVs. For preparation of BBMVs from superficial and juxtamedullary nephrons, kidneys were sliced horizontally in 3 mm sections. The outer 3 mm

Abbreviations: BBM, brush-border membrane; PTH, parathyroid hormone; BBMv, brush-border membrane vesicle; OK, opossum kidney; TPTX, thyroparathyroidectomized; PKC, protein kinase C; 1,25(OH) $_2$ D $_3$, 1,25-dihydroxyvitamin D $_3$.

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Figure 1 P_i transport in renal BBMVs isolated from TPTX and sham-operated rats

Uptake of P_i by kidney-cortical BBMVs from TPTX and sham-operated ('Sham-ope') rats was measured at 20 °C for the indicated times in the presence of Na^+ . Data are means $\pm$ S.E.M. for six rats. * $P < 0.05$; ** $P < 0.01$ versus sham-operated rats.

portion of the cortex (superficial cortex) and the inner cortex (juxtamedullary cortex), including the outermost portion of red medulla, were used for preparation of BBMVs [12].

Preparation of BBMVs and transport assay

BBMVs were prepared from rat kidney cortex by the Ca^{2+} -precipitation method as described previously [13]. The purity of the membranes was assessed by measuring leucine aminopeptidase, Na^+ , K^+ -ATPase, and cytochrome *c* oxidase activities

[14]. Uptake of [^{32}P] P_i was measured by a rapid-filtration technique [14]. Transport assay was initiated by the addition of 10 μ l of vesicle suspension to 100 μ l of incubation solution [100 mM NaCl/100 mM mannitol/20 mM Hepes/Tris (pH 7.4)/0.1 mM $KH_2^{32}PO_4$]. After incubation at 20 °C, transport was terminated by rapid dilution with 3 ml of ice-cold stop solution [100 mM mannitol/20 mM Hepes/Tris (pH 7.4)/0.1 mM KH_2PO_4 /20 mM $MgSO_4$ /100 mM choline chloride] and the resulting mixture was immediately transferred to a pre-moistened filter (0.45 μ m pore size) maintained under vacuum. After washing, filter-associated radioactivity was determined by liquid-scintillation spectroscopy.

Northern-blot analysis

Total RNA was isolated from kidney cortex by extraction with acid guanidinium thiocyanate/phenol/chloroform as described previously [15]. Total RNA was denatured at 70 °C for 5 min in a solution containing 10 mM Mops, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde and 50% (v/v) formamide, and subjected to electrophoresis in a 1.5% (w/w)-agarose gel containing 2.2 M formaldehyde. Resolved RNA was transferred to a Hybond- N^+ membrane (Amersham) and then covalently cross-linked by exposure to UV light. Hybridization was performed in a solution containing 50% formamide, 5 $\times$ SSPE [1 $\times$ SSPE is 0.15M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 2 $\times$ Denhardt's solution and 1% SDS. The membranes were analysed with a Fujifilm BAS-2000 system. An NaPi-2 cDNA probe was prepared as described previously [9].

Generation of peptide-specific antibodies and immunoblot analysis

Antibodies against rat NaPi-2 were generated in rabbit by injecting a peptide (Leu-Ala-Leu-Pro-Ala-His-His-Asn-Ala-Thr-Arg-Leu) corresponding to residues 626–637 located in the putative C-terminal intracellular domain of the protein [8,9]. An N-terminal cysteine residue was introduced for conjugation with keyhole-limpet haemocyanin (Sigma, St. Louis, MO, U.S.A.) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. The conjugates (100 μ g of peptide) were mixed with Freund's com-

Table 1 Effect of PTH on renal cortical Na^+ -dependent P_i co-transport activity in TPTX rats

P_i transport activity in the presence of Na^+ was measured in BBMVs isolated from sham-operated or TPTX rats 2 and 4 h after PTH injection. Data are means $\pm$ S.E.M. ($n = 7$). (a) * $P < 0.05$, ** $P < 0.01$ versus sham-operated; (b) * $P < 0.05$, ** $P < 0.01$ versus TPTX; (c) ** $P < 0.01$.

(a)			
Group	P_i uptake (nmol/30 s per mg of protein) (%)	Protein (%)	mRNA (%)
Sham-operated	0.64 ± 0.02 (100)	100	100
TPTX	1.34 ± 0.11 (21)**	350 ± 20 **	180 ± 40 *
(b)			
	P_i uptake (nmol/30 s per mg of protein) (%)	Protein (%)	mRNA (%)
TPTX	1.34 ± 0.11 (100%)	100	100
TPTX + PTH (2 h)	0.96 ± 0.31 * (73%)	56 ± 18 **	110 ± 15
TPTX + PTH (4 h)	0.76 ± 0.21 * (58%)	34 ± 10 **	115 ± 20
(c)			
	V_{max} (pmol/15 s per mg of protein)	K_m (mM)	
TPTX rats	1359 ± 342	0.12 ± 0.02	
+ PTH (4 h)	842 ± 166 **	0.11 ± 0.02

Figure 2 Effects of PTH on the amounts of NaPi-2 protein and mRNA in the kidney cortex of TPTX rats

Renal BBMVs (50 μ g of protein) were subjected to immunoblot analysis with the antibodies to rat renal NaPi-2 protein or a neutral-and-basic-amino-acid transporter (NBAT) protein. Total RNA (20 μ g) from kidney cortex was subjected to Northern-blot analysis with 32 P-labelled cDNA probes for rat NaPi-2 mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Lane 1, sham-operated control; lane 2, TPTX rat; lanes 3 and 4, TPTX rats 2 and 4 h respectively after PTH injection.

Table 2 Effect of dietary P_i on renal Na^+/P_i co-transport activity in TPTX and sham-operated rats

Sham-operated and TPTX rats were fed a control- P_i or low- P_i diet for 2 weeks. Some animals fed the low- P_i diet were given a high- P_i diet and killed 2 or 4 h later. Data are means $\pm$ S.E.M. ($n = 6$). $^{1**}P < 0.01$ versus corresponding control- P_i , $^{2*}P < 0.05$, $^{2**}P < 0.01$ versus corresponding low- P_i control.

Diet	P_i uptake (nmol/30 s per mg of protein)	
	Sham-operated	TPTX
Control- P_i	0.67 ± 0.13	1.25 ± 0.18
Low- P_i	$1.22 \pm 0.34^{1**}$	1.47 ± 0.43
Low- P_i	1.05 ± 0.15 (100%)	1.47 ± 0.43 (100%)
+ High P_i (2 h)	0.68 ± 0.21 (65%) 2**	1.13 ± 0.34 (77%) 2*
+ High P_i (4 h)	0.54 ± 0.15 (51%) 2**	0.77 ± 0.27 (52%) 2**

plete adjuvant and injected subcutaneously into rabbits four times at 2-week intervals. The antibodies were affinity-purified with the antigenic peptide immobilized on Cellulofine AM (Seikagaku Kogyo, Tokyo, Japan) [9]. Rat renal proximal BBMVs were prepared as described above in the presence of protease inhibitors and subjected to SDS/PAGE. Separated proteins were transferred electrophoretically to a nitrocellulose filter as described previously [16].

Immunohistochemistry

Under sodium pentobarbital anaesthesia, rats were transcardially perfused with saline followed by 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Kidneys were removed,

Table 3 Effect of dietary P_i on the amount of renal Na^+/P_i co-transport protein in TPTX or sham-operated rats

Results are means $\pm$ S.E.M. ($n = 6$). $^{1*}P < 0.01$ versus corresponding control. $^{2*}P < 0.05$, $^{2**}P < 0.01$ versus corresponding low- P_i control.

Diet	Amount of renal Na^+/P_i co-transport protein (%)	
	Sham-operated	TPTX
Control	100	100
Low P_i	$450 \pm 20^{1**}$	$140 \pm 13^{1*}$
Low P_i	100	100
+ High P_i (2 h)	$55 \pm 13^{2**}$	$76 \pm 21^{2**}$
+ High P_i (4 h)	$47 \pm 10^{2**}$	$60 \pm 18^{2**}$

immersed in the fixative for 15 h, and processed for preparation of cryostat sections. After microwave irradiation (for 5 min in 10 mM citrate buffer, pH 6.0) and treatment with H_2O_2 , the sections were incubated overnight at 4 $^\circ\text{C}$ in the NaPi-2 antibodies (1:2000 dilution) [9]. Immunoreaction was revealed with a Cy3-labelled goat anti-rabbit IgG (Chemicon, Temecula, CA, U.S.A.), and observed under a confocal laser scanning microscope TCS-4D (Leica, Bensheim, Germany).

Statistical analysis

Data were expressed as means $\pm$ S.E.M. and were analysed by one-way analysis of variance and Student's t test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Uptake of P_i by BBMVs from TPTX rats

P_i uptake by renal-cortical BBMVs isolated from TPTX and sham-operated rats was linear for 30 s in the presence of Na^+ (Figure 1). In the absence of Na^+ , an overshoot in P_i uptake was not observed (results not shown). The extent of P_i uptake at 30 s in BBMVs from TPTX rats was ≈ 2.1 -fold of sham-operated animals. As shown in Table 1, injection of PTH into TPTX rats resulted in decreases in Na^+/P_i co-transport activity within 4 h. Na^+ -dependent amino acid or glucose-transport activities were unchanged after PTH treatment (results not shown). Kinetic analysis indicated that the PTH-induced decrease in P_i uptake in TPTX rats was due to a decrease in V_{max} rather than to an increase in K_m (Table 1c).

Effects of PTH on the amounts of NaPi-2 mRNA and protein in the kidney cortex of TPTX rats

Immunoblot analysis of BBMVs with antibodies to NaPi-2 (Figure 2) revealed a protein of 90 kDa [9]. No positive band was obtained by the preabsorbed antibody with the peptide [9]. In the amount of NaPi-2 protein in BBMVs, there was a 3.5-times increase in TPTX rats compared with sham-operated animals (Table 1). Administration of PTH to the TPTX animals resulted in a marked decrease in the abundance of NaPi-2 immunoreactive protein within 4 h, whereas the level of a neutral-and-basic-amino-acid transporter protein was unchanged (Figure 2). The NaPi-2 mRNA level was about 1.8-fold higher in TPTX rats than in the sham-operated control (Figure 2). After admini-

Figure 3 Effect of PTH on NaPi-2 immunoreactivity in superficial cortex of TPTX rats

Immunostaining of NaPi-2 in the kidney cortex of TPTX (**B**) and sham-operated (**A**) rats. NaPi-2 immunoreactivity was more abundant in the apical membrane of superficial nephrons of TPTX rats (**B**) than sham-operated rats (**A**). NaPi-2 immunoreactivity was decreased in the superficial nephrons of TPTX rats 2 h after injection with PTH (**C**). The apical membranes of superficial nephrons of the TPTX rats are stained by the the antibody (**D**), but not by the preabsorption antibody with the antigen peptide (**E**). The bars represent 10 or 20 μ m.

stration of PTH, no changes in NaPi-2 mRNA levels were shown in the TPTX rats (Table 1).

Effects of dietary P_i on P_i transport, the amounts of NaPi-2 mRNA and protein in the kidney cortex of TPTX rats

P_i transport activity in BBMVs from TPTX rats fed the low- P_i diet for 2 weeks was similar to the corresponding value for the group fed the control- P_i diet (Table 2). In TPTX rats fed the low-

P_i diet, switching to the high- P_i diet rapidly decreased in the P_i transport activity. The amounts of NaPi-2 protein were slightly increased in TPTX rats fed the low- P_i diet (Table 3). In the TPTX rats fed the low- P_i diet, switching to the high- P_i diet resulted in decrease of 40% in the amount of the NaPi-2 protein within 4 h. Similar observations were obtained from sham-operated animals (Table 3). In contrast, dietary P_i did not affect the amounts of NaPi-2 mRNA in TPTX and sham-operated animals (results not shown).

Figure 4 Effect of PTH on NaPi-2 immunoreactivity in juxtamedullary cortex of TPTX rats

Small amounts of NaPi-2 immunoreactivity were apparent in the juxtamedullary cortex in sham-operated rats (A). NaPi-2 immunoreactivity was markedly increased in the juxtamedullary cortex of TPTX rats (B) and disappeared 4 h after injection with PTH (C). The bars represent 10 μ m.

Effects of dietary P_i on P_i transport in the superficial and juxtamedullary cortex

BBMVs were prepared from the superficial and juxtamedullary cortex of the TPTX rat kidney. P_i uptake remained linear for up to 30 s in both types of vesicles (results not shown). The initial rate of P_i uptake was greater in BBMVs from the superficial cortex than in those from the juxtamedullary cortex of the sham-operated rats. In the TPTX animals fed the low- P_i diet, the initial rate of P_i uptake in BBMVs from the superficial cortex was increased slightly (Table 4). At 4 h after switching to the high- P_i diet, the initial rate of P_i uptake was 20 and 72% of values for the TPTX animals fed the low- P_i diet for BBMVs from the superficial and juxtamedullary cortex respectively (Table 4).

Effects of PTH and dietary P_i on the localization of NaPi-2 immunoreactivity in the kidney of the TPTX rat

NaPi-2 immunoreactivity was seen in the apical membrane of proximal-tubular epithelial cells in sham-operated animals

(Figures 3A and 4A). In the renal cortex of TPTX rats, NaPi-2 immunoreactivity was much stronger in both superficial and juxtamedullary nephrons (Figures 3B and 4B). In PTH-injected TPTX rats, NaPi-2 immunoreactivity largely diminished in the apical membranes of both the nephrons (Figures 3C and 4C). No positive staining was obtained by the preabsorbed antibody with the peptide (Figures 3D and 3E).

In TPTX rats, NaPi-2 immunoreactivity was not affected with feeding of a low- P_i diet for 2 weeks (Figures 5A and 5B). After feeding of the high- P_i diet in P_i -deprived TPTX rats, NaPi-2 immunoreactivity was decreased to an undetectable level in superficial nephrons at 4 h, but was unchanged in juxtamedullary nephrons (Figures 5C and 5D).

DISCUSSION

In acute phase, the two stimuli dietary P_i and PTH regulate the endocytotic/exocytotic pathway in renal proximal tubules as described previously [9,10]. In the present study, the amounts of NaPi-2 protein and mRNA in the kidney cortex of TPTX rats were increased compared with those in the sham-operated animals. Administration of PTH to the TPTX rats resulted in a rapid decrease in the amount of NaPi-2 protein, but not in the abundance of NaPi-2 mRNA. This effect of PTH is markedly decreased by inhibiting endocytosis with microtubule-disrupting agents [17]. These observations suggest that one mechanism by which PTH inhibits renal type II Na^+/P_i co-transport is by endocytotic removal of the transporter from the BBM of renal proximal tubules. This inhibitory effect is mediated by a PTH receptor [18,19], which has been detected in the basolateral membrane of renal proximal tubular cells [20]. The binding of PTH to its receptor activates multiple signalling pathways mediated by cAMP-dependent protein kinase, phospholipase C and protein kinase C (PKC), which may contribute to the inhibition of P_i transport [20]. Friedlander et al. [21,22] have shown that part of the PTH-generated cAMP acts on Na^+/P_i co-transport via a luminal mechanism involving re-uptake of adenosine at the BBM. In *Xenopus* oocytes expressing the rat type II Na^+/P_i co-transporter (NaPi-2), activation of PKC leads to the inhibition of this transporter function [21]. However, a mutant NaPi-2 protein from which the PKC consensus phosphorylation sites were removed by site-directed mutagenesis was still inhibited by PTH, suggesting that regulation of the type II Na^+/P_i co-transporter by PTH does not involve phosphorylation of these sites by PKC [23]. Moreover, it is not clear whether a final target in PTH-induced phosphorylation is the type II transporter itself, or associated proteins which mediate kinase-dependent regulation of its transport function [3].

In the P_i -deprived TPTX rats, feeding of the high- P_i diet resulted in marked decreases the amount of total renal NaPi-2 protein (Tables 3 and 4). Immunohistochemical analysis suggested that NaPi-2 expression of superficial nephrons was sensitive to the diet. In contrast, NaPi-2 immunoreactivity was equally affected in both superficial and juxtamedullary nephrons by PTH status.

An earlier study reported that both superficial and juxtamedullary BBM possesses high-affinity systems of P_i uptake with similar K_m [24]. These high-affinity systems clearly respond to the diet and PTH status by changing its V_{max} . In superficial BBM, however, PTH influenced the K_m value of these systems, whereas the diet did not. These authors concluded that PTH and dietary P_i influence differently P_i uptake by BBMVs from superficial and juxtamedullary nephrons [24]. In the present study, PTH did not affect K_m values in the BBMV isolated from superficial

Figure 5 Effect of dietary P_i on NaPi-2 immunoreactivity in the kidney cortex of TPTX rats

(A) NaPi-2 immunoreactivity in the superficial cortex of renal proximal tubules of TPTX rats fed a low- P_i diet for 2 weeks; (B) in the juxtamedullary cortex of same animals; (C) in the superficial cortex 4 h after eating of a high- P_i diet; (D) in the juxtamedullary cortex 4 h after eating of a high- P_i diet. The bar represents 10 μ m.

nephrons in TPTX rats. This discrepancy may be the difference in the experimental conditions (BBMV preparation, acute TPTX and dietary P_i content).

The present study suggested that dietary P_i could regulate expression of the type II Na^+/P_i co-transporter protein in the superficial nephron in a PTH-independent manner. However, the mechanism by which dietary P_i regulates the endocytic/exocytic pathway remains unknown. One possibility may be an elevation of the plasma levels of 1,25-dihydroxyvitamin D_3 [$1,25(\text{OH})_2\text{D}_3$], which regulates renal Na^+/P_i co-transport activity, because the dietary P_i level markedly affects the level of

plasma $1,25(\text{OH})_2\text{D}_3$. However, $1,25(\text{OH})_2\text{D}_3$ regulates the expression of NaPi-2 protein at the transcriptional level, but not in the endocytic/exocytic pathway [25]. One of other possibilities may be that the alteration of plasma P_i level directly modulates the endocytic/exocytic pathway. In OK cells a low P_i concentration in the medium results in an increase in apical Na^+/P_i co-transport activity, and increasing P_i concentration leads to lowering of P_i uptake [26], suggesting that apical P_i influx regulates Na^+/P_i co-transport activity in OK cells. *In vivo*, apical P_i influx may modulate the endocytic/exocytic pathway of the type II Na^+/P_i co-transporters. However, further study is

Table 4 Effect of dietary P_i on the amount of renal Na⁺/P_i co-transport activity in juxtamedullary and superficial cortex from TPTX rats

P_i transport activity was determined for the superficial and juxtamedullary cortex as in Table 2. Results are means ± S.E.M. (*n* = 4). ¹**P* < 0.05 versus corresponding sham-operated animals. ²**P* < 0.05, ²***P* < 0.01 versus corresponding TPTX (low-P_i).

Diet	P _i uptake (nmol/30 s per mg of protein)	
	Superficial	Juxtamedullary
Sham-operated	0.76 ± 0.12	0.57 ± 0.11
TPTX	1.23 ± 0.32 ¹ *	1.12 ± 0.25 ¹ *
TPTX (low-P _i)	1.45 ± 0.33 (100%)	1.04 ± 0.13 (100%)
+ High P _i (2 h)	0.45 ± 0.11 (31%) ² **	0.79 ± 0.11 (75%) ² *
+ High P _i (4 h)	0.29 ± 0.03 (20%) ² **	0.75 ± 0.18 (72%) ² *

needed to clarify the regulation of the endocytic/exocytic pathway by dietary P_i.

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