ORIGINAL

Effect of dietary components on renal inorganic phosphate (Pi) excretion induced by a Pi-depleted diet

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Abstract : Dietary inorganic phosphate (Pi) is the most important factor in the regulation of renal Pi excretion. Recent studies suggest the presence of an enteric-renal signaling axis for dietary Pi as well as the existence of a mechanism by which the intestine detects changes in luminal Pi concentrations. The mechanisms of intestinal Pi sensing, however, are unknown. In the present study, we focused on Pi depletion signals and investigated the effects of dietary components on intestinal Pi sensing. After feeding rats experimental diets for 3 days, we investigated urinary Pi excretion and plasma biochemical parameters. Renal Pi excretion was suppressed in rats fed a low-Pi diet (0.02% Pi). Elimination of dietary calcium (Ca) completely blocked the suppression of Pi excretion, suggesting that the presence of Ca is essential for the Pi depletion signal. Furthermore, a minimum Ca content of more than 0.02% was necessary for the Pi depletion signal. Magnesium, lanthanum, and strontium, which are agonists of calcium sensing receptor, instead of Ca, reduced Pi excretion. Therefore, dietary Ca appears to be important for the Pi depletion-sensing mechanism in the gastrointestinal tract. In addition, the calcium sensing receptor may be involved in the Pi depletion signal. J. Med. Invest. 61 : 162-170, February, 2014

Keywords **:** *dietary phosphate, sensing, calcium, phosphate excretion*

1.INTRODUCTION

Inorganic phosphate (Pi) retention is a major harmful complication of chronic kidney disease (CKD), leading to secondary hyperparathyroidism and ectopic calcification, and is significant risk for cardiovascular morbidity and mortality (1-3). High-normal serum Pi levels are associated with cardiovascular events and mortality among individuals having normal kidney function (4). Therefore, restricting dietary Pi is necessary to prevent hyperphosphatemia followed by a progression of serious complications.

Dietary Pi is the most important factor in body Pi homeostasis (5, 6). Dietary Pi levels control active vitamin D synthesis, parathyroid hormone (PTH) secretion, and renal Pi reabsorption (7-9). Renal Pi reabsorption is a key determinant of serum Pi levels in the body (10, 11). A low-Pi diet can lead to almost 100% renal reabsorption of filtered Pi, whereas a high-Pi diet leads to decreased proximal tubular P_i reabsorption (10, 11). The factors controlling the dietary adaptive system are not known, but do not include PTH, vitamin D, growth hormone, thyroid

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hormone, calcitonin, or fibroblast growth factor-23 (12, 13).

Dietary Pi restriction in humans and animals is associated with enhanced renal tubular Pi reabsorption (14). Urinary excretion of Pi declines within hours after a reduction in dietary Pi intake, and Pi virtually disappears from the urine within 1 to 2 days. Changes in renal tubular reabsorption of Pi and its urinary excretion may occur before a fall in the plasma concentration of Pi becomes evident (14). This renal response to Pi depletion develops in normal, parathyroidectomized, and vitamin D-deficient animals (14). These reports suggest the presence of an enteric-renal signaling axis for Pi, and also imply the existence of a mechanism by which the intestine detects changes in luminal Pi, independent of known phosphaturic factors (14, 15). It is not known, however, which dietary components, e.g., sucrose, protein, or calcium (Ca), affect intestinal dietary Pi sensing. Using a rat model to monitor renal Pi excretion induced by a low-Pi diet, we investigated the essential dietary components for Pi depletion signals in the gastrointestinal tract.

2. MATERIALS AND METHODS

2.1. Animal and Diets

Rats were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of Tokushima University School of Medicine. Male 6-week-old Wistar rats were purchased from Charles River Laboratories Japan (Yokohama, Japan). The rats were provided free access to water and standard rat chow (Oriental, Osaka, Japan). Before dietary adaptation, the rats were fed a control diet (CP, 0.6% Pi, 0.6% Ca) based on modified AIN93G for 6 days. Thereafter the animals were fed test diets (Studies 1-3).

All experimental diets were based on the AIN93G rodent diet. Egg white was used as the protein source instead of casein (16). An AIN93G mineral mixture without Pi and Ca sources was used to adjust the concentration of Ca and Pi for each test diet.

For study 1, the rats were divided into six groups ; a control Pi (CP) diet, and low-Pi (LP) diets 1 through 5. Rats were placed on one of the following diets for 3 days. The CP diet included an AIN93G-based diet without protein, AIN93G mineral mixture without Pi and Ca sources, 20% egg white (protein source), sucrose (a carbohydrate source), KH_2PO_4, and $CaCO_3$. Pi levels and Ca levels in the CP diet were adjusted to 0.6% using KH_2PO_4 and $CaCO_3$, respectively. The LP1 diet was the same as the CP diet, except the Pi source (K_2HPO_4) was eliminated (final concentration : 0.02% Pi and 0.6% Ca). The LP2 diet was the same as the LP1 diet, except the Pi source (egg white) was eliminated (final concentration : 0% Pi and 0.6% Ca). The LP3 diet was the same as the LP2 diet, except the Pi and Ca sources (K_2HPO_4, $CaCO_3$, and egg white) were eliminated (final concentration : 0% Pi and 0% Ca). The LP4 diet was the same as the LP3 diet, except the sucrose was removed (final concentration : 0% Pi and 0% Ca). The LP5 diet was the same as the LP4 diet, except Ca was added (final concentration : 0% Pi and 0.6% Ca). The final concentrations of Pi and Ca are shown in Table 1.

Table 1 Comparison of the experimental diet for study 1

	Egg white	KH_2PO_4	$CaCO_3$	Sucrose	Final % of P	Final % of Ca
CP	+	+	+	+	0.6	0.6
LP1	+	-	+	+	0.02	0.6
LP2	-	-	+	+	0	0.6
LP3	-	-	-	+	0	0
LP4	-	-	-	-	0	0
LP5	-	-	+	-	0	0.6

For study 2, the rats were divided into six groups (CP) diet, and Ca-modified low-Pi (CaLP) diets 1 through 5. Rats were fed one of the following diets for 3 days. CP is described above. In CaLP1, the Pi source (K_2HPO_4, and egg white) was eliminated from the CP diet (final concentration : 0% P and 0.6% Ca). In CaLP2, CaLP3, and CaLP4, the Pi source (K_2HPO_4, and egg white) was also eliminated from the CP diet. Ca levels in the CaLP2 diet were adjusted to 0.3% using $CaCO_3$ (final concentration : 0% Pi and 0.3% Ca). Ca levels in the CaLP3 diet were adjusted to 0.15%. Ca levels in the CaLP4 diet were adjusted to 0.02%. In the CaLP5 diet, both the Pi and Ca sources (K_2HPO_4, $CaCO_3$, and egg white) were eliminated from the CP diet (final concentration : 0% Pi and 0% Ca). Final concentrations of Pi and Ca are shown in Table 2.

For study 3, the rats were divided into five groups, each fed a mineral-modified 0% Pi diet. A Ca (0.6% Ca), no Ca (-Ca diet, 0% Ca), magnesium (Mg), lanthanum (La), or strontium (Sr) diet. Mg, La,

Table 2 Comparison of the experimental diet for study 2

	Egg white	Kh2PO4	CaCO3	Sucrose	Final % of P	Final % of Ca
CP	+	+	+	+	0.6	0.6
CaLP1	-	-	+	+	0	0.6
CaLP2	-	-	+	+	0	0.3
CaLP3	-	-	+	+	0	0.15
CaLP4	-	-	+	+	0	0.02
CaLP5	-	-	-	+	0	0

and Sr levels were adjusted to 0.6% using $MgCO_3$, $La_2(CO_3)_3$, and $SrCO_3$, respectively. Rats were fed one of the test diets for 3 days. Final concentrations of Pi and Ca in the diets are shown in Table 3.

Table 3 Comparison of the experimental diet for study 3

	Egg white	KH_2PO_4	$CaCO_3$	Sucrose	Final % of P	Final % of Ca	
-Ca	-	-	-	+	0	0	
Ca	-	-	+	+	0	0.6	
Mg	-	-	-	+	0	0	0.6% of Mg
La	-	-	-	+	0	0	0.6% of La
Sr	-	-	-	+	0	0	0.6% of Sr

2.2. Concentrations of plasma Ca, Pi, and PTH

Concentrations of plasma or urinary Pi, and Ca were determined using commercial kits (Wako, Osaka, Japan) (16). Concentrations of plasma PTH were determined using the PTH ELISA kit (Immunotopics Inc., San Clemente, CA) (16). Tail vein blood collection was performed using a 1-mL syringe with a 25-gauge needle at 11 : 00 AM for all studies.

Metabolic cages were used collect urine, and urine pools from rats over a 24-h period were analyzed on the last day of each study (16).

2.3. Statistical Analysis

Data are expressed as means ± SE. Statistical analysis was performed using two-factor factorial analysis of variance. A P-value of less than 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Study 1 ; Effect of low-Pi diets on plasma and urinary Pi levels.

After feeding the rats each diet for 3 days, the biochemical parameters were analyzed in the plasma and urine of the animals (Fig. 1). Food intake did not differ among groups (data not shown). The LP1 (0.02% Pi and 0.6% Ca) and LP2 groups (0% Pi and 0.6% Ca) showed hypophosphatemia and a markedly reduced urinary Pi excretion compared with the CP group (0.6% Pi and 0.6% Ca ; Fig. 1A and 1B). The LP3 (0% Pi and 0% Ca) and LP4 (deletion of sucrose, 0% Pi and 0% Ca) groups showed normal plasma Pi levels and Pi excretion, as did the CP group. In contrast, the LP5 group (deletion of sucrose, 0% Pi and 0.6% Ca) showed hypophosphatemia and hypophosphaturia (Fig. 1A and 1B). In the absence of Ca, the low-Pi diet did not affect the levels of plasma Pi and urinary Pi excretion (Fig. 1A and 1B). Plasma Ca levels were not significantly different among any of the groups (Fig. 1C). The LP1, LP2, and LP5 groups showed hypercalciuria compared with the CP group (Fig. 1D). Elimination of dietary sucrose did not affect the plasma Pi and urinary Pi levels. In addition, plasma PTH levels were significantly reduced after feeding on the LP1 or LP2 diet (Fig. 1E). Compared with LP1 and LP2, plasma PTH levels were significantly increased in animals fed the LP3 diet. These data suggest that dietary Ca levels are an essential factor for the Pi depletion signal in the diet.

3.2. Study 2 ; Effect of Ca in a low-Pi diet on Pi excretion

To further investigate the effect of Ca concentration in a low-Pi diet, we prepared low-Pi diets containing various concentrations (0.6~0%) of Ca (CaLP1-CaLP5 ; Fig. 2). The amounts of food intake did not differ among any of the groups (data not shown). Animals were fed low-Pi diets with 0.6% (CaLP1), 0.3% (CaLP2), 0.15% (CaLP3), 0.02% (CaLP4), and 0% (CaLP5) Ca for 3 days. Intake amounts of 0.6% (CaLP1), 0.3% (CaLP2), 0.15% (CaLP3), and 0.02% Ca (CaLP4) significantly decreased plasma Pi levels compared with the CP diet (Fig. 2A). The Pi-free diets containing 0% Ca (CaLP5), however, did not show hypophosphatemia. In contrast, urinary Pi excretion levels were suppressed in the CaLP1-5 groups compared with those in the CP group (Fig. 2B). Furthermore, the urinary Pi excretion levels were significantly higher

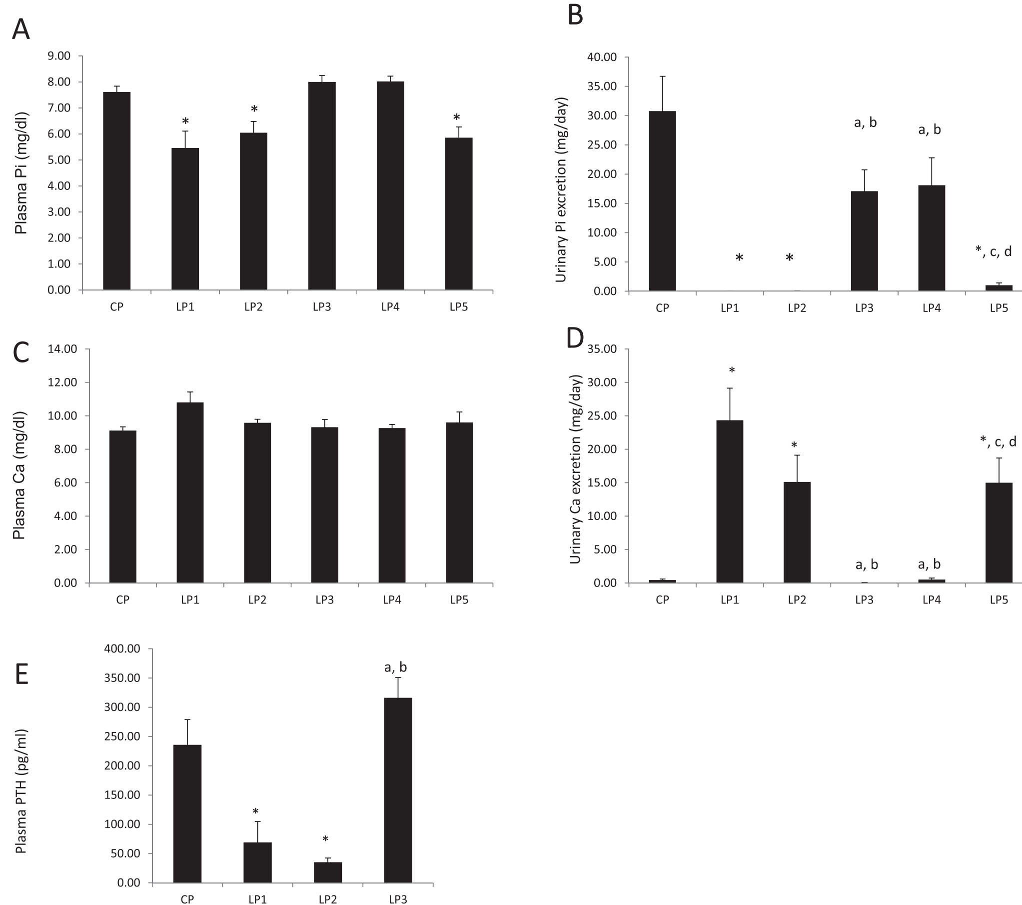

Figure 1 Effect of various diets on plasma and urinary Pi levels
Three days after adaptation to the test diet, concentrations of plasma Pi, Ca, and PTH were determined in tail vein blood. Rats were placed in a metabolic cage to collect urine for 24 h on the last day of the study. (A) Plasma Pi, (B) urinary Pi excretion, (C) plasma Ca, (D) urinary Ca excretion, and (E) plasma PTH levels in rats after feeding with a control (CP), low-Pi (LP)1, LP2, LP3, LP4, or LP5 diet (n=3-4), The study was repeated three times. Values are mean ± SE. $^{*}p < 0.05$ versus CP, $^{a}p < 0.05$ versus LP1, $^{b}p < 0.05$ versus LP2, $^{c}p < 0.05$ versus LP3, and $^{d}p < 0.05$ versus LP4.

in the CaLP4 and CaLP5 groups compared with the CaLP1-3 group. Plasma Ca levels were not significantly different among any of the groups (Fig. 2C). In the CaLP5 group, urinary Ca excretion was significantly increased compared with the other groups (Fig. 2D). The CaLP4 group had increased urinary Pi excretion, whereas plasma Pi levels were reduced. This reason for these findings is unclear. Urinary Pi excretion is considered an excellent marker of Pi depletion. In this context, we suggest that at least 0.02% Ca content in the diet is essential for the signal of Pi depletion.

3.3. Study 3. Effect of CaR agonist in a low-Pi diet on Pi excretion

To examine the effects of various metal ions as substitutes for Ca, we investigated the effect of Mg, La, and Sr, which are Ca sensing receptor (CaR) agonists, on the Pi depletion signal (17, 18). In this experiment, food intake did not differ among the five groups (data not shown). The -Ca diet (0% Pi and 0% Ca) did not result in hypophosphatemia or hypophosphaturia, as described above (Fig. 1, Fig. 3A, and 3B). The Ca diet (0% Pi and 0.6% Ca) led to hypophosphatemia and hypophosphaturia, whereas the Mg diet (0% Pi and 0.6% Mg), the La diet (0% Pi and 0.6% La), and the Sr diet (0% Pi and 0.6% Sr) did not lead to hypophosphatemia (Fig. 3A and 3B). Urinary Pi excretion levels were slightly but significantly decreased in rats on the Mg diet (Fig. 3B). Similar observations were obtained for the La (Fig. 3A and 3B). The Sr diet did not significantly decrease the urinary Pi excretion compared with CP diet (Fig. 3B). Among all groups, we observed no significant changes in plasma Ca levels (Fig. 3C). The Ca diet-fed animals exhibited hypercalciuria, but not the other diet groups (Fig. 3D). We then investigated plasma PTH levels after feeding of these diets. In animals fed the Ca diet (0.6% Ca, 0% Pi), plasma PTH levels were markedly decreased

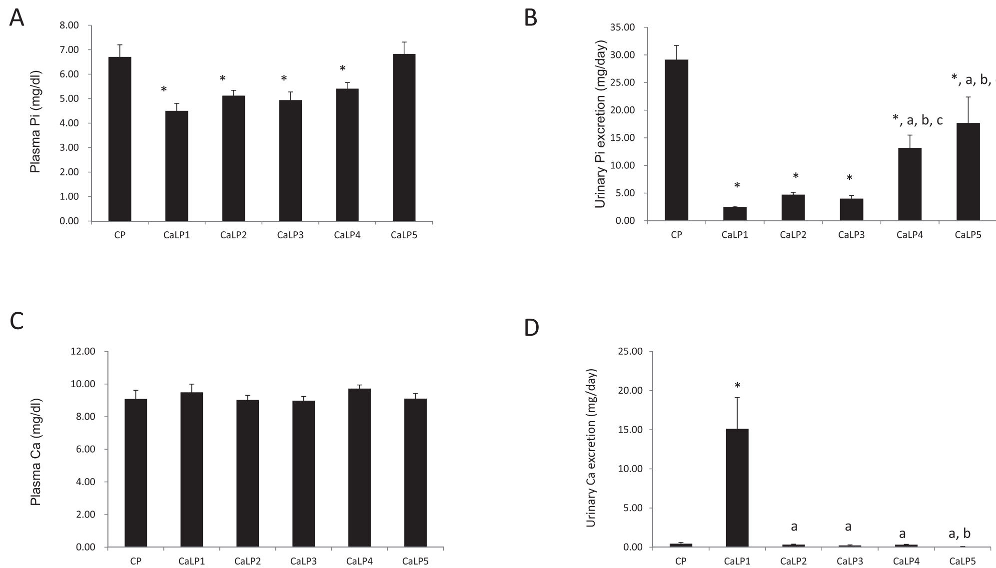

Figure 2 Effect of Ca concentration in a low-Pi diet on Pi metabolism
Three days after adaptation to test diet, concentrations of plasma Pi and Ca were determined in tail vein blood. Rats were placed in a metabolic cage to collect urine for 24 h on the last day of the study. (A) Plasma Pi, (B) urinary Pi excretion, (C) plasma Ca, and (D) urinary Ca excretion levels in rats after feeding with a control (CP), or calcium modified low phosphorus (CaLP) 1-5 diet (n=3-4). The study was repeated three times. Values are mean ± SE. $*p < 0.05$ versus CP, $^{a}p < 0.05$ versus CaLP1, $^{b}p < 0.05$ versus CaLP2, $^{c}p < 0.05$ versus CaLP3

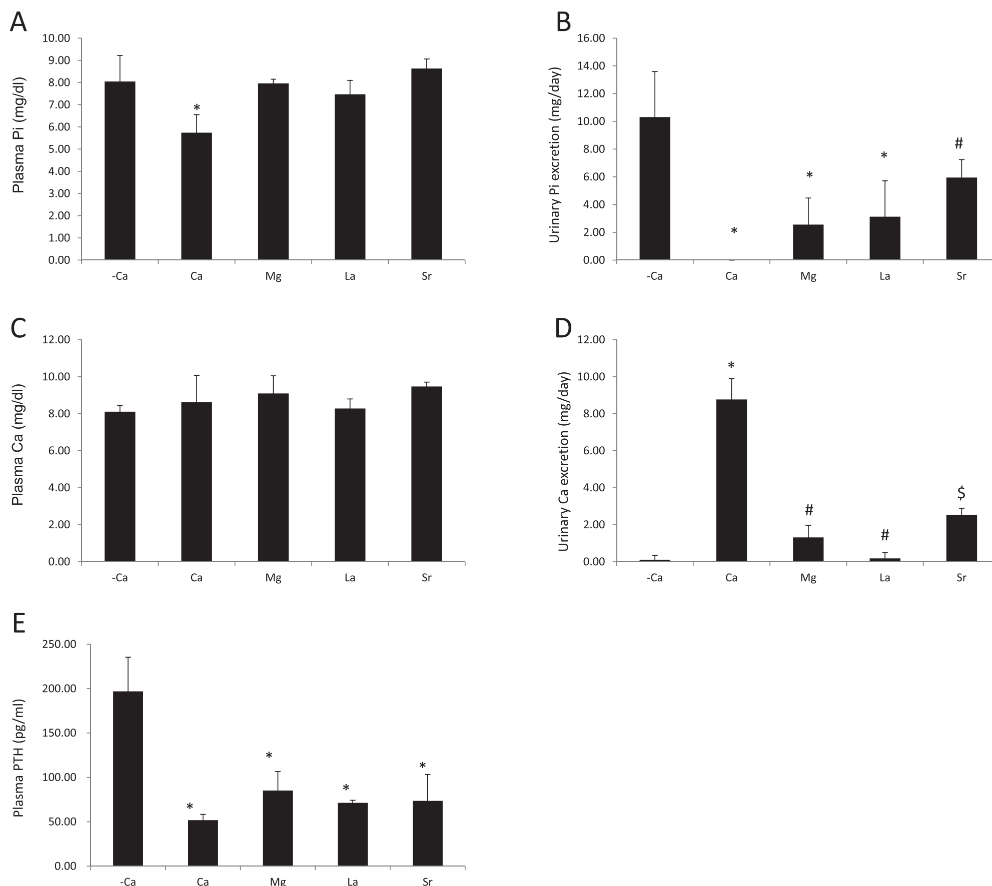

Figure 3 Effect of divalent ions in a low-Pi diet on Pi metabolism
To examine various metal ions in substitution for Ca, we prepared test diets : magnesium (Mg), lanthanum (La), or strontium (Sr) diets. Three days after adaptation to the test diet, concentrations of plasma Pi, Ca, and PTH were determined in tail vein blood. Rats were placed in a metabolic cage to collect urine for 24 h on the last day of the study. (A) Plasma Pi, (B) urinary Pi excretion, (C) plasma Ca, (D) urinary Ca excretion, and (E) plasma PTH levels in rats after feeding on a -Ca diet (0% Pi and 0% Ca), and phosphorus-depletion diets including Ca (0% Pi and 0.6% Ca), Mg (0% Pi and 0.6% Mg), La (0% Pi and 0.6%La), or Sr (0% Pi and 0.6% Sr) (n=3-4). This study was repeated three times. Values are mean ± SE. $*p < 0.05$ versus -CP, $^{\#}p < 0.05$ versus Ca, $^{\$}p < 0.05$ versus La.

compared with those in animals fed the -Ca diet (Fig. 3E). In animals fed the Mg, La and Sr diet, plasma PTH levels were also significantly lower than those fed the -Ca diet. These data suggest that Mg, La, and Sr partially recover Pi depletion signals in the absence of Ca.

4. DISCUSSION

To clarify the sensing of dietary Pi restriction, we prepared several diets modified for each component (mineral, protein, and carbohydrate), and analyzed renal Pi excretion. In the present study, we prepared five different diets with modified carbohydrate (sucrose), protein, or mineral contents, and found that luminal Ca concentration is an important factor for Pi sensing. Pi restriction diets affect urinary Pi and Ca excretion, plasma PTH, vitamin D, and fibroblast growth factor-23 levels (19). It is not clear whether these measures are true indexes of Pi depletion. Urinary Pi excretion, however, is considered the most important index associated with dietary Pi intake (20-22).

In the present study, using urinary Pi excretion as an index of Pi depletion, we showed that more than 0.02% Ca is essential for the recognition of a Pi-restricted diet. In human studies, dietary Ca is normally plentiful when food is plentiful, so that the soluble Ca ion concentration in the lumen is ~5 to 10 mM after a meal (23). In animals fed a diet containing 0.6% Ca, the soluble Ca ion concentration in the intestinal lumen is ~5 to 10 mM (24). In the Ca-restricted diet (0.02-0.15%), a concentration of less than 1 mM Ca in the lumen was essential for signaling Pi restriction.

Although low levels (< 1 mM Ca) of luminal Ca might be involved in the Pi depletion signal in the small intestine, it is not clear whether extracellular Ca or transported Ca is involved in sensing in the epithelial cells. It is widely accepted there are two pathways of Ca absorption, one transcellular and the other paracellular (23, 24). Transcellular absorption in a saturable process occurring at a luminal concentration up to approximately 5 mM, whereas paracellular absorption displays linear or diffusive kinetics between 5 and 200 mM Ca^{2+}. The transcellular process involves three major steps : entry across the brush border, mediated by a TRPV6 ; intracellular diffusion, mediated by calbindin D9k ; and extrusion, mediated largely by PMCA1b (24). This pathway is regulated by $1,25(OH)_2D_3$ through the vitamin D receptor. In a previous study, we investigated the signals of dietary Pi restriction in vitamin D receptor-null mice, but the signals were preserved in vitamin D receptor-null mice (25), suggesting that transcellular Ca transport signals are not likely involved in sensing Pi restriction.

In contrast, nutrients are detected in the lumen of the intestine by chemosensors positioned in both absorptive epithelial cells and enteroendocrine cells where they transduce nutritional information to the body (26). Much has been learned from studies of lingual nutrient detection due to identification of the G protein family, which couples with nutrient-sensitive G protein coupled receptors located in the taste buds on the tongue and in the intestine (26, 27). The CaR, a G protein-coupled receptor, is best known and understood for its control of PTH synthesis and secretion in response to changes in Ca, Mg, Sr, and La (17, 18). CaRs are distributed along most of the gastrointestinal tract, from the stomach to the large intestine (23, 28). The physiologic role of CaRs is the maintenance of constant blood Ca^{2+} levels (1.1-1.3 mM) through continuous adjustments of PTH release from the parathyroid chief cells, which are highly sensitive to the slightest changes in extracellular Ca ion concentrations (17, 18). In the present study, we showed that Mg, La, or Sr, which are CaR agonists, can partially substitute for dietary Ca. In this context, these data suggest that Ca sensing exists and is necessary for recognition of low Pi.

In contrast, for sensing a high-Pi diet, evidence in support of a Pi entero-renal axis also comes from a very recent and convincing study in which Pi or saline were acutely administered in the rat duodenum ; this study was performed in normal (intact) and thyroparathyroidectomized rats and after renal denervation (15). Intestinal, but not gastric, Pi administration results in a prompt increase in Pi excretion, which is independent of PTH and renal nerve activity. The authors excluded changes in circulating fibroblast growth factor-23 or frizzled related protein-4 and demonstrated that homogenates of the duodenal mucosa also shows increased Pi excretion (15). We performed similar studies and concluded that the presence of Ca in the high Pi diet was not essential for the dietary Pi signal (data not shown). The existence of Ca is not necessary for recognition of the high Pi diet, but Ca is necessary for recognizing a low-Pi diet. Therefore, it is likely that the supposed sensing mechanism of the Pi depletion signal differs from that for sensing a high-Pi

diet (29).

In the present study, we investigated the levels of plasma PTH after feeding of various diets. Martin *et al.* reported that dietary Pi acutely controls PTH release by the parathyroid glands by a mechanism independent of changes in plasma Ca, but that may involve concomitant changes in plasma Pi (30). On the other hand, the ability of phosphonoformate (phosphate analogue) to acutely increase PTH levels in the absence of changes in plasma Ca and Pi provides evidence for a signal derived from the gastrointestinal tract that can trigger PTH release (30). We examined whether PTH-dependent Pi excretion is involved in Pi restriction signals, but the results did not provide a clear answer. Further studies are needed to clarify the mechanisms of PTH secretion by Pi restriction signals.

The characteristics of the Pi sensor molecules in the intestine are still unknown. We suggest that the CaRs play a role in dietary Pi recognition. Indeed, previous studies demonstrated that activation of CaRs in enteroendocrine G-cells and I-cells stimulates the secretion of gastrin and cholecystokinin, respectively (31, 32). In addition, CaR detects L-amino acids within the concentration ranges of the diets (33, 34). Depletion of extracellular Ca ion diminishes the secretion of gluco-insulinotropic peptide, glucagon-like peptide-1, and peptide tyrosine tyrosine in response to L-amino acids (34). Thus, the extracellular Ca ion concentration may determine the magnitude of the gut peptide secretion response. In the present study, we suggest that CaRs are important regulators of Pi sensor activity.

Animal and human studies of CKD suggest that dietary Pi restriction could mitigate nephron loss, curb secondary hyperparathyroidism, and lower blood pressure (35-38). Pi binders are widely used to control hyperphosphatemia in clinical CKD; however, none of the currently available binders is ideal (39, 40). An alternative approach to the use of binders to limit hyperphosphatemia in CKD is to target the Pi intestinal transporter or sensor directly (39). Identifying the Pi sensor molecules is a potential new target for controlling hyperphosphatemia.

Finally, using urinary Pi excretion as an index of Pi depletion, we investigated the Pi-sensing mechanisms for the Pi restriction diet. Dietary Ca concentration (0.02% Ca) is a critical parameter for Pi depletion in the gastrointestinal tract. In addition, Ca receptor agonists may improve the Pi-sensing mechanism. Further studies are needed to identify the target molecule for sensing a Pi restriction signal.

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