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Paracellin-1 is critical for magnesium and calcium reabsorption in the human thick ascending limb of Henle

Anne Blanchard, Xavier Jeunemaitre, Philippe Coudol, Michèle Dechaux, Marc Froissart, Adrien May, Renato Demontis, Albert Fournier, Michel Paillard, and Pascal Houillier

Université Pierre et Marie Curie, INSERM U356, Institut Fédératif de Recherche 58, Laboratoire de Génétique Moléculaire, Hôpital Universitaire Européen Georges Pompidou, Hôpital Universitaire Necker, and Assistance Publique-Hôpitaux de Paris, Paris; Centres Hospitaliers Généraux Evry and Creil; and Hopital Universitaire, Amiens Sud, France

Paracellin-1 is critical for magnesium and calcium reabsorption in the human thick ascending limb of Henle.

Background. A new protein, named paracellin 1 (PCLN-1), expressed in human thick ascending limb (TAL) tight junctions, possibly plays a critical role in the control of magnesium and calcium reabsorption, since mutations of PCLN-1 are present in the hypomagnesemia hypercalciuria syndrome (HHS). However, no functional experiments have demonstrated that TAL magnesium and calcium reabsorption were actually impaired in patients with HHS.

Methods. Genetic studies were performed in the kindred of two unrelated patients with HHS. Renal magnesium and calcium reabsorption in TAL were analyzed in one homozygous affected patient of each family, one patient with extrarenal hypomagnesemia (ERH), and two control subjects (CSs).

Results. We found two yet undescribed mutations of *PCLN-1* (Gly 162 Val, Ala 139 Val). In patients with HHS, renal magnesium and calcium reabsorptions were impaired as expected; NaCl renal conservation during NaCl deprivation and NaCl tubular reabsorption in diluting segment were intact. Furosemide infusion in CS markedly increased NaCl, Mg, and Ca urinary excretion rates. In HHS patients, furosemide similarly increased NaCl excretion, but failed to increase Mg and Ca excretion. Acute $MgCl_2$ infusion in CS and ERH patient provoked a dramatic increase in urinary calcium excretion without change in NaCl excretion. When combined with $MgCl_2$ infusion, furosemide infusion remained able to induce normal natriuretic response, but was unable to increase urinary magnesium and calcium excretion further. In HHS patients, calciuric response to $MgCl_2$ infusion was blunted.

Conclusion. This study is the first to our knowledge to demonstrate that homozygous mutations of *PCLN-1* result in a selective defect in paracellular Mg and Ca reabsorption in the TAL, with intact NaCl reabsorption ability at this site. In addition, the study supports a selective physiological effect of basolateral Mg^{2+} and Ca^{2+} concentration on TAL divalent cation paracellular permeability, that is, PCLN-1 activity.

Key words: nephrocalcinosis, hypomagnesemia-hypercalciuric syndrome, extrarenal hypomagnesemia, NaCl reabsorption, claudin 16, renal transport.

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Magnesium balance is regulated by kidneys that adapt magnesium excretion to net magnesium intestinal absorption. In experimental models, the thick ascending limb of Henle (TAL), and especially the cortical TAL, is the main site of magnesium transport, accounting for 60 to 70% of overall magnesium reabsorption [1]. In this tubular segment, magnesium transport is mainly (if not exclusively) passive via the paracellular pathway, and is driven by the prevailing lumen-positive transepithelial potential difference generated by transcellular NaCl reabsorption [2]. Thus, two conditions are required for the paracellular magnesium reabsorption in the cortical TAL. The transepithelial voltage must be oriented lumen positive, and the paracellular pathway must be able to conduct divalent cations. Regulatory events on divalent cation reabsorption may be indirectly related to transepithelial NaCl reabsorption via transepithelial voltage generation. However, growing experimental data suggest that peptide hormones and basolateral concentration of divalent cation may regulate Mg and Ca transport in cortical TAL without a change in transepithelial voltage, suggesting that a selective effect on paracellular permeability exists [1–4]. The paracellular pathway is also a site for sodium transport, but transport of monovalent and divalent cations may be dissociated [3, 4]. Whereas the cellular mechanisms involved in transcellular active NaCl reabsorption and transepithelial voltage generation have been well described, until recently little was known about the molecular nature of the TAL paracellular pathways.

Recently, Simon et al have identified a new protein, named paracellin-1 (PCLN-1) or claudin 16, belonging

to the claudin protein family [5]. This protein is expressed in human TAL tight junctions and might play a critical role in the control of paracellular permeability for magnesium and calcium. Indeed, homozygous mutations in the *PCLN-1* gene are associated with a rare familial disease, the hypomagnesemia hypercalciuria syndrome (HHS), which is secondary to marked renal magnesium and calcium wasting [reviewed in 6]. Thus, it has been proposed that PCLN-1 is a key in TAL divalent cation handling. However, to date there are no functional data demonstrating that TAL magnesium and calcium reabsorptions are actually impaired in patients presenting with HHS and mutations in the *PCLN-1* gene. The rationale for the present study was that if PCLN-1 is critical for selective divalent cation paracellular permeability in the TAL, then a loss-of-function of PCLN-1 is expected, first to induce a defect in renal magnesium and calcium handling without affecting NaCl renal transport under baseline conditions. Second, maneuvers that normally inhibit divalent cation reabsorption in TAL, that is, furosemide and acute magnesium infusions, should be unable to decrease further divalent cation reabsorption at this site in HHS patients.

This study describes the clinical and biological characteristics of two families bearing two new mutations (Gly-162Val, Ala139Val) in *PCLN-1* as well as the results of dynamic investigations conducted in the two unrelated probands, one hypomagnesemic patient with extrarenal hypomagnesemia (ERH), and two control subjects (CSs). First, patients with genetically identified HHS have an abnormally low renal magnesium and calcium reabsorption without renal loss of NaCl. Second, the normal effects of acute furosemide infusion on calcium and magnesium excretions were blunted in HHS patients, in spite of normal natriuretic and chloruretic effects. In addition, acute magnesium infusion failed to increase calcium excretion in these patients, as it did in ERH patient and CSs. To our knowledge, these data provide the first evidence for functionality of the mutations described and for a major role of PCLN-1 in paracellular divalent cation reabsorption in human TAL. In addition, in normal subjects, acute $MgCl_2$ infusion induced a calciuric response without a change in NaCl excretion, and when combined with furosemide infusion, the calciuric response was blunted but natriuretic response to furosemide was conserved. These data suggest a selective inhibitory effect of hypermagnesemia on paracellular divalent cation permeability in human TAL, probably via a modification of PCLN1 activity.

METHODS

Patients

Families of HHS patients. Two unrelated consanguineous kindreds (one from Portugal and one from Morocco) whose probands had typical HHS were investigated. After informed consent was obtained from each participating subject (or the parents of the younger children), a basal clinical and biological analysis was performed. All subjects were analyzed in France for screening evaluation in the Nephrology Units of Louise Michel (Evry) and Laennec (Creil) General Hospitals or of Amiens University Hospital. Appropriate venous blood and urine samples were drawn for evaluation of blood creatinine, Ca, Mg, phosphate, and urinary creatinine and Ca excretion.

More extensive baseline and dynamic studies were performed in the two probands of each family in the Departments of Physiology of Broussais and Necker University Hospitals in Paris. Twenty-four–hour urines were collected the day before the baseline studies and were analyzed for Ca, Mg, Na, K, creatinine, and citrate. Appropriate fasting urinary (120-minute collection period) and venous blood samples were analyzed for urinary creatinine, Ca, Mg, phosphate and blood creatinine, Na, Cl, K, total CO_2, pH, total and ionized Ca, Mg, phosphate, PTH 1-84, 25-hydroxy-vitamin D and 1,25-dihydroxy-vitamin D, active renin, and aldosterone.

Urinary concentration ability was assessed by 1-desamino-[D-Arg^8] (dDAVP) administration [7] and acidification ability by an acute oral load of either NH_4Cl or $NaHCO_3$ [8]. The ability to reclaim filtered sodium chloride renal handling was studied by either the ability to lower sodium excretion under sodium diet deprivation or assessment of fractional chloride reabsorption in the diluting segment under conditions of maximal free water clearance obtained by hypotonic saline infusion, as previously described [9, 10].

Genotyping. DNA was extracted from blood sample according to the classic phenol extraction method. The search for mutation was performed by amplification and direct sequencing of each of the five exons of the *PCLN-1* gene (Genbank accession NM-006580) using the primers described by Simon et al [5].

Double-strand sequencing was performed using the dideoxy-termination method on an ABI 377 instrument (Prism Big Dye™ Terminators Cycle Sequencing kit; PE Applied Biosystems, Foster City, CA, USA). Each individual of the two kindred was genotyped by amplification and specific allele detection test. The Ala139Val (GCG→GTG) mutation located at exon 2 creates a *BstX*I site with an amplicon size of 183 bp for wild-type individuals and amplicons of 136 and 47 bp for homozygous-affected subjects. Since the Gly162Val (GGAGTA) mutation located at exon 3 does not modify any restriction enzyme site, we used the mutagenically separated polymerase chain reaction (PCR) technique where both normal and mutant alleles are amplified in the same tube, using different length allele-specific primers [11]. The following primers were designed with one forward primer and two

reverse primers where additional deliberate differences (underlined) were introduced to correspond to the molecular variant and to reduce cross reactions between the two alleles: Ex3F, 5′-TGTCTGCCCATGTTGCC ATCCTG-3′; Ex3RSGly, 5′-GATATTCTAGCTGGG TTTAG-3′; and Ex3RLVal, 5′-GCGTTGATGATTA CTGCAAGTATTCTAGCTGGGTTCGT-3′.

Polymerase chain reactions were conducted in a 25 μL reaction volume containing 2.5 μL 10 × PCR buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.3, 0.01% gelatin), 2.0 mmol/L $MgCl_2$, 10 μmol/L each of the four dNTPs, 5 pmol each of the three primers and 1 UAmpliTaq Gold DNA polymerase (Roche Molecular System, USA). The first denaturation step (94°C for 5 minutes) was followed by 35 cycles: 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The amplification reaction yielded 161 and 179 bp products for the Gly and Val alleles, respectively, which were resolved on a 2% agarose gel.

Two groups of 96 normal volunteers and 96 subjects with idiopathic hypercalciuria were genotyped, using the PCR detection assays set up for both mutations, which did not reveal any positive individual.

Patient with extrarenal hypomagnesemia (ERH). A 40-year-old woman was referred to our laboratory for episodic tetany. Explorations revealed hypoparathyroidism caused by severe magnesium depletion of extrarenal origin. Hypomagnesemia persisted despite a 3 g/day oral magnesium supplementation. Further investigations led to conclude that she had a selective intestinal defect in magnesium absorption. This patient was submitted to acute and chronic magnesium infusion.

Control subjects. Two 30-year-old control women without history of osteoporosis, lithiasis, and renal failure were studied.

Physiological studies: Protocol investigation

During all of the tests, fasting patients and controls were recumbent but were allowed to stand to void. All maintained their usual diet with a normal NaCl intake the days before exploration. For these studies, urine was generally collected at 30-minute intervals, and venous blood was drawn through an indwelling catheter at the midpoint of each period.

Glomerular filtration rate (GFR) measurements. In all studies, GFR was assessed through a continuous inulin analog infusion method. A priming dose of polyfructosan S (Inutest®), 30-mg/kg body weight, was injected intravenously, followed by a constant Inutest® infusion (15 mg/min/100 mL estimated GFR). Baseline urine collections were initiated after a one-hour equilibration period.

Acute and chronic intravenous $MgCl_2$ infusion. After two 30-minute control periods, four additional 30-minute urine collections were obtained during continuous $MgCl_2$ infusion (25 mmol in 5% dextrose over two hours) for measurement of urinary inulin, Ca and Mg, and serum inulin, total and ultrafilterable (UF) Ca and Mg. For chronic $MgCl_2$ infusion, 40 mmol/day $MgCl_2$ was infused after completion of the acute infusion over the 48 following hours.

Furosemide infusion. After two 30-minute control periods, a priming dose of furosemide (0.2 mg/kg body weight) was injected intravenously, and then followed by an infusion of 10 mg/hour in 0.9% saline solution at constant rate over two hours. After a single one-hour equilibration period, two 30-minute urine collections were performed. In all periods, urinary inulin, Na, Cl, Ca and Mg, and serum inulin, Na, Cl, total and UF Ca and Mg were measured.

Analytical methods

Analytical methods used were described and referenced in a previous article [12]. Plasma and urine concentrations of inulin were measured according to the Schreiner's method, pH and P_{CO2} using an automated pH and gas analyzer (ABL 330, Radiometer, Copenhagen), creatinine by a colorimetric method (picric acid), Ca and Mg by atomic absorption spectrophotometry (Perkin Elmer, Model 3110), Na and K by specific electrodes (Beckman, model E2A), and phosphorus and urine citrate by colorimetry. Serum-ionized Ca was measured by an ion-selective electrode (ICA 2, Radiometer, Copenhagen), the PTH concentration by a radio-immunometric method (Allegro PTH; Nichols Institute, San Juan Capistrano, CA, USA), plasma 25-hydroxy-vitamin D by Preece's method and 1,25-dihydroxy-vitamin D by radioreceptor assay, plasma renin and aldosterone radioimmunoassay kits (Pasteur Diagnostics, France, and Behring GmbH, respectively). Serum UF Ca and UF Mg were obtained by a micropartition system (MPS 1; Amicon, Beverly, MA, USA). Furosemide was obtained from Hoechst (Paris, France), and inulin analog polyfructosan-S (Inutest®) from Laevozan GmbH (Linz, Austria).

Calculations

Urinary Ca and Mg excretion variations could be due to GFR variations affecting the Ca and Mg filtered loads. Thus, we factored urinary Ca and Mg excretion values by GFR in order to assess the tubular Ca and Mg reclamations independently of GFR values. The fractional excretion of calcium and sodium (FE_{Ca}/FE_{Na}) ratio was used to discriminate between selective effects on tubular Ca reclamation that dissociate Ca and Na excretion and nonselective effects similarly affecting Ca and Na reabsorption [13].

RESULTS

Basal genotype-phenotype relationship

The two unrelated HHS consanguineous kindred (kindred A from Portugal, kindred B from Morocco) were

Table 1. Basal biological characteristics of the two hypomagnesemia hypercalciuric syndrome (HHS) kindreds

	Kindred A					Kindred B					
	IA-1	IA-2	IIA-1	IIA-2	IIA-3	IB-1	IB-2	IIB-1	IIB-2	IIB-3	IIB-4
Age *years*	56	54	31	25	21	45	39	16	12	10	8
Sex *male/female*	M	F	M	F	F	M	F	F	F	F	M
Nephrocalcinosis	−	−	−	+	−	−	−	−	+	−	−
Plasma											
Calcium *mmol/L*	2.20	2.31	2.19	2.02	2.36	2.25	2.30	2.29	2.20	2.34	2.35
Magnesium *mmol/L*	0.69	0.79	0.71	0.39	0.67	0.76	0.68	0.83	0.61	0.85	0.81
Phosphate *mmol/L*	1.14	1.14	0.96	1.33	0.76	0.81	0.96	1.24	1.33	1.45	1.58
Creatinine *μmol/L*	137	74	78	112	84	76	48	49	62	41	70
Urine											
Calcium excretion											
mmol/mmol creatinine	0.54	0.55	0.17	0.67	0.45	0.30	0.60	0.07	1.39	0.18	0.15
Status	+/−	+/−	+/−	+/+	+/−	+/−	+/−	−/−	+/+	−/−	+/−

Nephrocalcinosis scores are: + presence, − absence. Status scores are: +/+ affected, +/− carrier, −/− normal.

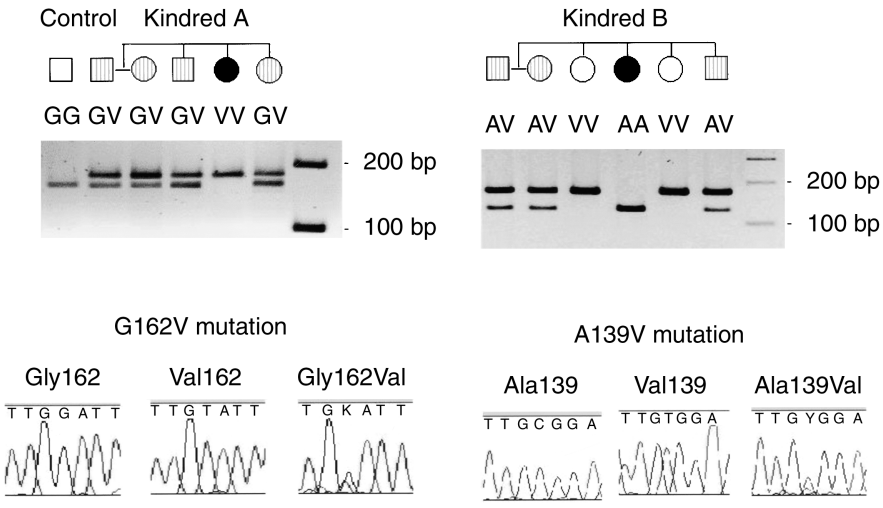

Fig. 1. The G162V and A139V mutations of the paracellin-1 gene and their segregation in two hypomagnesemia hypercalciuric syndrome (HHS) families. (*A*) HHS families. Symbols refer to affected individuals (●), carriers (vertically striped circles and squares), and healthy individuals (○, □). The segregation of each mutation is shown by an allele-specific PCR assay (**Methods** section). (*B*) Electropherograms documenting each type of mutation are shown in a trio composed (from left to right) of a control, an affected subject, and a heterozygous carrier. All sequences shown are in the forward direction with respect to paracellin-1 mRNA.

investigated through two probands who had typical features of HHS. The proband of kindred A (subject IIA-2) was a 25-year-old woman with hypercalciuria, bilateral nephrocalcinosis, and hypomagnesemia (Table 1). The proband of kindred B (subject IIB-2) was a 12-year-old girl with similar features. Both had a medical history of constant growth retardation (−2 SD). The first-degree relatives of both patients were asymptomatic.

Direct sequencing of the *PCLN1* gene revealed that the proband of kindred A was homozygous for a missense mutation Gly162Val (GGA→GTA) located at exon 3 and that the proband of kindred B was homozygous for another new missense mutation Ala139Val (GCG→GTG) located at exon 2 (Fig. 1). These mutations were analyzed through PCR amplification followed by a BstXI enzymatic digestion for the A139V mutation or by specific MS-PCR for the G162V mutation. Genotypes confirmed the heterozygosity of each parent. Siblings of each affected proband were either heterozygous carrier or unaffected. PCR detection assays set up for both mutations did not reveal any positive individual out of 96 normal volunteers.

Urinary calcium excretions and serum calcium concentrations in the relatives were within the normal ranges. However, heterozygous first relatives had a tendency toward hypercalciuria or mild hypomagnesemia (Table 1).

Biological characteristics of the probands and ERH patients

HHS patients had a defect in renal magnesium and calcium reabsorption, but no renal loss of sodium. To demonstrate the defect in renal magnesium and calcium reabsorption, baseline data in HHS patients were compared with those obtained in one patient with extrarenal hypomagnesemia (Table 2).

In HHS patient 1 (subject IIA-2 with HHS), the severe reduction in serum Mg concentration was associated with a moderate reduction in serum total and ionized Ca concentrations (Table 2). Patient 2 (subject IIB-2 with HHS) had moderate hypomagnesemia and a normal cal-

Table 2. Phenotypical evaluation of the patients with hypomagnesemia hypercalciuria syndromes (HHS 1 and 2) and one patient with extrarenal hypomagnesemia (ERH)

	HHS 1	HHS 2	ERH	Normal values[a]
Age	25	12	40	
Body weight *kg*	102	45	61	
Height *m*	1.51	1.43	1.60	
GFR $mL \cdot min^{-1}$	50	60	113	
(normal values)	(81–132)	(89–165)	(70–118)	
Blood				
Creatinine *µmol/L*	112	71	54	60–105
Calcium *mmol/L*	2.02	2.24	1.84	2.17–2.57
Ultrafiltrable calcium *mmol/L*	1.22	1.53	1.17	1.34–1.62
Ionized Ca^{2+} *mmol/L*	1.05	1.22	0.97	1.14–1.35
Magnesium *mmol/L*	0.39	0.58	0.37	0.71–1.04
Phosphate *mmol/L*	1.22	1.39	1.10	0.80–1.40
TmPi *mmol/L*	1.14	1.64	1.08	adult: 0.75–1.37 child: 1.47–2.07
Sodium *mmol/L*	139	137	140	135–145
Potassium *mmol/L*	4.3	3.8	4.1	3.5–4.5
Chloride *mmol/L*	98	104	100	95–105
Blood pH	7.38	7.37	7.35	7.37–7.42
Total CO_2 *mmol/L*	29	29	27.4	24–30
Plasma				
Renin *pg/mL*	21	22	NA	15–50
Aldosterone *pg/mL*	239	251	NA	30–360
24-hour urine *mmol/24 h*				
Calcium	8.0	8.9	0.2	
Magnesium	3.0	3.9	0.2	
Sodium	155	62	134	
Potassium	72	31	35	
Citrate	0.30	0.98	NA	>1.67
Maximum U_{Osm} after dDAVP *mOsm/kg* H_2O	360	510	NA	>850
Urinary acidification defect	distal	distal	NA	
Distal fractional chloride reabsorption	84–88%	NA	NA	>75%
Adaptation to low NaCl diet	NA	conserved	NA	

Abbreviations are: UF, ultrafilterable; TmPi, renal tubular maximum phosphate; dDAVP, 1-desamino-[D-Arg^8]; NA, not available; GFR, glomerular filtration rate.

[a]Normal values from our laboratory

cium concentration. The patient with ERH presented as the HHS1 patient with a severe Mg depletion and low Ca concentration.

In the ERH patient, urinary magnesium and calcium excretion were very low and appropriate to the low serum magnesium and calcium concentrations. These data in ERH patient are in agreement with data observed in experimental human magnesium depletion [14]. In contrast, in the HHS1 patient with similar severe Mg depletion, despite the similarly low serum Ca value, urinary magnesium and calcium excretion was much higher and thus inappropriate. In the HHS patient 2 with moderate Mg depletion, the serum Ca concentration was normal, but urinary Ca and Mg excretions were also inappropriately high. Thus, renal tubular Mg and Ca reabsorption was markedly decreased in HHS patients.

In patients with HHS, levels of circulating renin and aldosterone were normal, suggesting normal blood and extracellular volume. In addition, HHS patient 2 was normally able to lower her sodium excretion below 10 mmol/day during sodium deprivation, and in HHS patient 1, sodium reabsorption in the diluting segment was normal as assessed by hypotonic saline infusion. Indeed, whatever the index considered, the distal fractional chloride reabsorption was normal: $C_{H_2O}/(C_{H_2O} + C_{Cl})$ values ranged between 84% and 88%, which stand within the normal range (>75%) in our laboratory and others [9]. In addition, C_{H_2O}/GFR values were normal with regard to the distal delivery $(C_{H_2O} + C_{Cl})$/GFR, according to the reference frame previously established in normal subjects receiving various NaCl intakes [10]. These data suggest that PCLN-1 mutations did not alter tubular NaCl reabsorption.

In addition, inulin clearance (GFR) was abnormally low in both patients with HHS. This moderate renal failure was associated with mild proteinuria (<1 g/24 h) without glycosuria or abnormal aminoaciduria. In both patients, maximal urinary osmolality values reached after dDAVP administration were abnormally low. Plasma acid-base values, estimated from plasma pH and total CO_2 values, were normal. However, in patient 1, urinary P_{CO_2} remained close to venous P_{CO_2} after acute oral $NaHCO_3$ load, whereas renal tubular maximum (Tm) for bicarbonate was normal (30 mmol/L GFR; normal value range is 23 to 30). Patient 2 failed to have a decrease in urinary pH and increase in net proton excre-

Table 3. Effects of furosemide infusion in patients with hypomagnesemia hypercalciuria syndrome (HHS 1 and 2) and control subjects (CS 1 and 2)

	Furosemide	HHS 1	HHS 2	CS 1	CS 2
GFR *mL/min*	−	53	58	80	79
	+	51	56	84	66
Serum UF Mg *mmol/L*	−	0.69	0.50	0.59	0.58
	+	0.67	0.44	0.54	0.56
Serum UF Ca *mmol/L*	−	1.39	1.44	1.31	1.38
	+	1.41	1.48	1.32	1.34
Urinary Mg *mmol/L GF*	−	0.51	0.07	0.05	0.04
	+	0.51	0.09	0.10	0.15
Urinary Ca *mmol/L GF*	−	0.16	0.15	0.02	0.04
	+	0.20	0.16	0.18	0.24
Urinary Na *mmol/L GF*	−	2.71	2.23	2.69	3.80
	+	22.41	18.87	13.66	20.74
Urinary Cl *mmol/L GF*	−	3.84	3.30	2.82	3.93
	+	24.67	21.16	15.87	23.33

Results are the means of two 30-minute baseline collection periods (−) or of the two final 30-minute collection periods (+) during furosemide infusion. Abbreviations are UF, ultrafilterable; GFR, glomerular filtrate rate.

tion after oral NH_4Cl load: Minimal urinary pH was 5.8 (normal value <5.4), and maximal net acid excretion reached only 24 μmol/min (normal value >80). Both subjects had hypocitraturia. The latter data suggested in the two probands distal defect of urinary acidification, probably related to nephrocalcinosis.

Magnesuric and calciuric, but not natriuretic, responses to furosemide were blunted in patients with homozygous mutations of the *PCLN-1* gene

Furosemide is well known to inhibit transcellular NaCl reabsorption in TAL, which in turn suppresses the transepithelial potential difference and thus inhibits Ca, Mg, and Na reabsorption through the paracellular pathway. If PCLN-1 is critical for paracellular magnesium and calcium reabsorption in TAL but not for paracellular Na reabsorption at this site, and if PCLN-1 mutations are responsible for loss of function of the protein, then furosemide can be expected in HHS patients, first, to normally inhibit NaCl reabsorption and increase NaCl excretion. Second, it would be unable to increase further calcium and magnesium excretion (because calcium and magnesium reabsorption is already suppressed). Furosemide infusions were performed in two CSs and in the two patients with HHS (Table 3). Patient 1 was studied after a 48-hour $MgCl_2$ infusion in order to normalize her filtered load of calcium and magnesium.

Before the furosemide infusion, serum ultrafilterable (UF) Ca concentrations were similar in patients with HHS and the controls. However, Ca excretion markedly differed and was approximately five times higher in HHS patients than in controls. $MgCl_2$-infused HHS patient 1 had a normalized serum UF magnesium concentration and a marked increase in magnesium excretion, while HHS patient 2 had a spontaneous low serum UF Mg concentration and conserved magnesium excretion. During furosemide infusion, Mg and Ca excretions increased approximately two- to ninefold, respectively, in CSs, whereas in HHS patients, the drug had almost no effect on Mg and Ca excretion. Finally, all subjects showed identical Na and Cl excretion under baseline conditions, and they were markedly and similarly increased approximately sixfold by furosemide.

These data obtained with furosemide in HHS patients indicate the presence of a defect in paracellular reabsorption of divalent cation in TAL (paracellular Na reabsorption remaining normal at this site), which is due to loss of function mutations in PCLN-1.

Acute $MgCl_2$ infusion elicited a calciuric, but not natriuretic, response in the normal subject and ERH patient

Experimental studies in rat, using in vivo microperfused Henle's loop, have shown that an acute increase in plasma magnesium concentration depressed absolute magnesium and calcium, but not sodium, absorption [15]. Because the filtered load of calcium but not the filtered load of magnesium remains unchanged during acute magnesium infusion in humans, the increase in calcium excretion is a better index of the inhibitory effect of peritubular magnesium on renal tubular divalent cation transport.

As shown in Table 4, acute $MgCl_2$ infusion in the CS and the patient with ERH induced a tenfold increase in urinary calcium excretion. As discussed previously in this article, these changes in calcium excretion occurred despite almost no change in serum UF Ca concentration and therefore reflected a decrease in renal calcium reabsorption. Urinary sodium excretion remained almost constant in both subjects during $MgCl_2$ infusion (data not shown). Accordingly, the FE_{Ca}/FE_{Na} ratio, which should remain constant if sodium reabsorption was primarily affected [13, 16], increased in the CS and ERH patient in proportion to urinary calcium excretion. These data

Table 4. Effects of $MgCl_2$ infusion on renal calcium handling in patients with hypomagnesemia hypercalciuria syndrome (HHS), one patient with extrarenal hypomagnesemia (ERH), and one control subject (CS)

	Baseline	1 hour	2 hours	48 hours
Serum UF Mg *mmol/L*				
HHS 1	0.24	0.65	1.07	0.69
HHS 2	0.49	0.70	1.31	
ERH	0.22	0.71	1.08	0.77
CS	0.61	1.02	1.36	
Serum, UF Ca *mmol/L*				
HHS 1	1.22	1.16	1.17	1.39
HHS 2	1.53	1.53	1.53	
ERH	1.21	1.17	1.16	1.36
CS	1.49	1.57	1.54	
Urinary Ca *mmol/L GF*				
HHS 1	0.12	0.17	0.18	0.16
HHS 2	0.12	0.23	0.23	
ERH	0.001	0.003	0.016	0.009
CS	0.02	0.12	0.20	
FE_{Ca}/FE_{Na}				
HHS 1	10.0	13.6	10.6	5.9
HHS 2	8.7	7.3	5.0	
ERH	0.1	0.2	1.2	0.3
CS	1.7	11.5	13.4	

Chronic $MgCl_2$ infusion was performed in HHS 1 patient and ERH patient. Abbreviations are: UF, ultrafilterable; FE, fractional excretion.

Table 5. Effects of furosemide infusion in one control subject first when normomagnesemic, and second when hypermagnesemic (after $MgCl_2$ infusion)

	GFR	Serum UF *mmol/L*		Urinary excretions *mmol/L GF*			
	mL/min	Ca	Mg	Ca	Mg	Na	Cl
Baseline	80	1.31	0.59	0.02	0.05	2.69	2.82
Furosemide	84	1.32	0.55	0.18	0.10	13.66	15.87
$MgCl_2$	82	1.38	1.09	0.11	0.45	3.06	4.04
$MgCl_2$ + furosemide	77	1.38	0.93	0.19	0.49	14.58	17.00

Abbreviations are: GFR, glomerular filtration rate; $MgCl_2$, $MgCl_2$ infusion; Furosemide, furosemide infusion; GF, glomerular filtrate.

indicate that an increase in plasma magnesium concentration also inhibits renal calcium reabsorption in humans via a mechanism independent of sodium reabsorption.

To localize the inhibitory effect of acute magnesium infusion on calcium reabsorption in renal tubules, furosemide infusion was repeated in one CS in whom hypermagnesemia was induced by $MgCl_2$ infusion (Table 5). $MgCl_2$ infusion induced a six- and tenfold increase in urinary Ca and Mg excretion, respectively, without any change in urinary NaCl excretion. During the subsequent furosemide infusion, urinary Ca excretion rose less than twofold (vs. 9-fold when normomagnesemic), and the magnesuric response was completely blunted, whereas the natriuretic response remained intact (Table 5). The lack of additive effects of $MgCl_2$ and furosemide infusions on divalent cation urinary excretion supports the concept in humans that the calciuric and magnesuric effects of $MgCl_2$ infusion are located in the TAL, which is

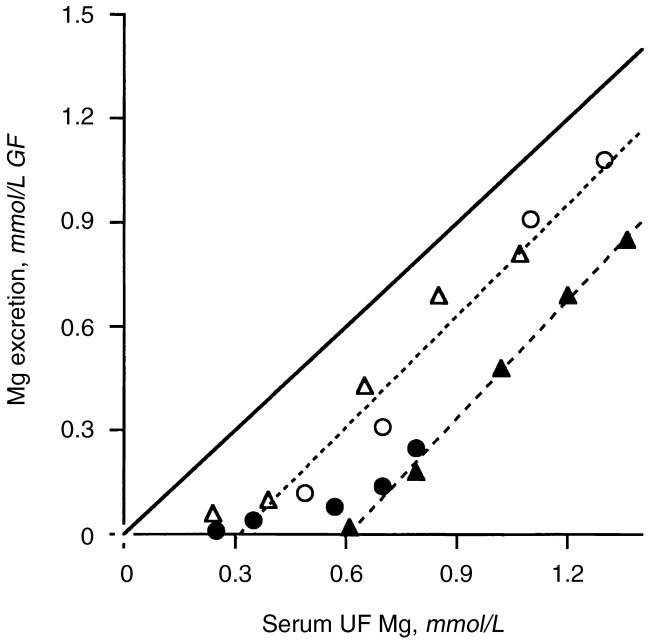

Fig. 2. Relationships between urinary magnesium excretion rate (factored by GFR) and ultrafilterable (UF) Mg serum concentration before and during acute infusion of $MgCl_2$ in the patients with hypomagnesemia-hypercalciuric syndrome (HHS; ○, △, dotted line), one patient with extrarenal hypomagnesemia (ERH; ●), and one control subject (CS; ▲, dashed line). The bisector on this figure (solid line) corresponds to the theoretical value of magnesium excretion if no magnesium is reabsorbed.

in agreement with previous data in animals. In addition, these results indicate that acute hypermagnesemia inhibits divalent cation reabsorption independently of Na reabsorption, suggesting a selective effect on paracellular pathway permeability.

The inhibitory effect of hypermagnesemia on TAL magnesium reabsorption in CSs contributes to the apparent saturation in renal magnesium reabsorption that is observed during acute magnesium infusion [17]. It has been shown in dogs and rats [15, 18] that the apparent saturation (or Tm) in renal magnesium reabsorption is not accounted for by a genuine saturable process but results from two components: proximal tubule Mg reabsorption, which increases with increasing luminal Mg concentration, and TAL Mg reabsorption, which is first enhanced due to increasing TAL Mg delivery and then declines because of the inhibitory effect of hypermagnesemia on the basolateral side of the epithelium. As shown in Figure 2, in acutely $MgCl_2$-infused CS, the relationship between urinary Mg excretion (factored by GFR) and serum UF Mg allowed calculation of an apparent Tm for renal Mg reabsorption of 0.56 mmol/L glomerular filtrate (GF) [17]. In the patient with ERH in whom serum UF Mg rose progressively, urinary Mg, initially close to zero, slowly rose until the normal serum UF Mg concentration was reached. Then it followed the same

relationship as the normal subject, indicating a normal apparent renal Tm for Mg.

Calciuric response to acute $MgCl_2$ infusion was blunted in patients with homozygous mutations of the *PCLN-1* gene

We have shown that acute $MgCl_2$ infusion inhibits Ca and Mg reabsorption in TAL without altering urinary NaCl excretion, suggesting that a selective inhibition of divalent cation paracellular pathway permeability exists. If PCLN-1 is critical for paracellular divalent cation reabsorption in TAL and if PCLN-1 mutations are responsible for loss of function of the protein, then in HHS patients $MgCl_2$ infusion should not be able to increase urinary Ca excretion further. Indeed, in HHS patients, the UF Ca concentration remained unchanged during acute $MgCl_2$ infusion, and urinary calcium excretion rates increased only slightly (less than 2-fold), while the FE_{Ca}/FE_{Na} ratio remained unchanged (Table 4). Thus, in HHS patients, TAL calcium reabsorption was poorly influenced by the $MgCl_2$ infusion. The blunted calciuric response to magnesium infusion was associated with a marked impairment in renal Mg handling. As shown in Figure 2, the relationship between urinary Mg excretion (factored by GFR) and serum UF Mg measured during $MgCl_2$ infusion allowed calculation of apparent Tm values for renal Mg reabsorption, which were abnormally low, approximately 0.30 mmol/L GF for both patients.

Taken together, the data obtained with $MgCl_2$ infusion in HHS patients confirm the presence of a selective defect in the paracellular pathway permeability to divalent cation in TAL, due to loss of function mutations in *PCLN-1*.

DISCUSSION

Renal magnesium wasting is usually defined as a urinary excretion greater than 1 mmol/day in the presence of hypomagnesemia (serum Mg <0.70 mmol/L). It may be related to drugs, to some endocrine disorders, and to relatively uncommon familial renal tubular defects [19]. In Bartter's and Gitelman's syndromes inherited as autosomal recessive traits, a renal Mg loss is associated with hypokalemia, metabolic alkalosis, and secondary hyperaldosteronism [reviewed in 20, 21]. In the HHS (classified MIM 248,250), the plasma potassium level is normal and metabolic alkalosis is absent. The cardinal clinical feature in HHS is the development of nephrocalcinosis, which is consistently associated with polyuria and occasionally with nephrolithiasis. Growth retardation may be observed, probably dependent on the nephrocalcinosis-related renal insufficiency. HHS generally progresses toward end-stage renal failure, and the presence of severe nephrocalcinosis is associated with a rapid alteration of renal function, as established in a recent follow-up study [6]. About nothing was known about the site and mechanism(s) of the renal defect(s) in HHS until Simon et al demonstrated homozygous mutations in the paracellin-1 gene, a newly described paracellular protein that is selectively expressed in human TAL [5].

We report the cases of two patients from two unrelated families; both issued from two full-cousin parents and presented with typical features corresponding to the HHS phenotype [6]. Genetic analysis was performed in the two families and two as yet undescribed homozygous mutations (Gly162Val, Ala139Val) of the paracellin-1 were found in the probands of each family. Heterozygous first relatives had a tendency to hypercalciuria or mild hypomagnesemia.

In the two patients with homozygous mutations in the *PCLN-1* gene, an impairment in renal tubular magnesium and calcium reabsorption with normal NaCl reclamation was demonstrated. Accordingly, comparative studies performed under baseline condition in one patient with ERH and in HHS patients demonstrated that the magnesium and calcium excretion in HHS patients were inappropriately high when compared with serum magnesium and calcium concentrations. However, renal NaCl reabsorption in HHS patients was intact. There was no clinical evidence of extracellular fluid volume contraction. Furthermore, basal circulating renin and aldosterone concentrations were normal and adapted to the normal Na intake. Finally, abnormal NaCl reclamation in the diluting segment of the nephron was excluded in one patient, while the other was able to adapt normally to a sodium deprived diet.

Two maneuvers were used to localize the defect in divalent cation reabsorption in the renal tubule in HHS patients: furosemide and $MgCl_2$ infusion.

The furosemide infusion was performed first. Assuming that HHS is due to an abnormally low paracellular pathway permeability for Mg and Ca in the TAL, NaCl being normally reabsorbed at this site, furosemide infusion is expected to induce a normal increase in NaCl excretion but to be unable to inhibit further the paracellular Mg and Ca reabsorption in TAL (which are already abolished). Therefore, any increase in Mg and Ca excretion observed in CSs after the furosemide infusion should be blunted in patients with HHS. Because Ca and Mg reabsorption in the TAL is directly related to Ca and Mg delivery, in order to obtain similar serum values to those of controls, extracellular Mg and Ca concentrations were normalized by chronic magnesium infusion in patient 1 with HHS. As expected, despite natriuretic and chloruretic responses to furosemide of similar magnitude in the patients with HHS and controls, the magnesuric and calciuric responses were blunted in the patients. Data obtained in the non-$MgCl_2$–infused patient (HHS patient 2) provide direct evidence that the abnormal

response to furosemide in patient 1 is not due to $MgCl_2$ infusion per se. This blunted response is likely due to an absence of Mg and Ca paracellular reabsorption in the TAL of patients with HHS. In addition, the normal natriuretic response to furosemide further supports a normal reabsorption of NaCl in the TAL of HHS patients.

Second, we used acute $MgCl_2$ infusion. Experimental data in rats have shown that an acute increase in plasma magnesium concentration selectively inhibits divalent cation reabsorption in the TAL [15]. The present study confirms that an acute $MgCl_2$ infusion also selectively inhibits divalent cation reabsorption in human TAL, as suggested by the absence of a natriuretic response, and confirmed by the absence of additive calciuric and magnesuric effects of acute $MgCl_2$ and furosemide infusion in one CS whereas the natriuretic effect of furosemide was conserved. In HHS patients, urinary calcium excretion was poorly influenced by $MgCl_2$ infusion, which further confirms that divalent cation paracellular reabsorption in the TAL is inhibited in these patients.

Our results showing a blunted divalent cation urinary response to two different maneuvers (furosemide and $MgCl_2$ infusion), which both inhibit divalent cation reabsorption in TAL of normal subjects, strongly support the presence of a primary defect in magnesium and calcium reabsorption at this site in HHS patients. In addition, NaCl reclamation in the diluting segment remains normal in these patients. Altogether, the results suggest that PCLN1 is essential for divalent cation but not for sodium paracellular permeability in the TAL.

A primary defect located in the distal convoluted tubule (DCT) was unlikely in HHS for at least two reasons. First, calcium and magnesium reabsorption in the DCT are active and mediated by distinct transcellular transport [22], rendering unlikely that a mutation of the *PCLN-1* gene results in a primary defect in tubular reabsorption of both divalent cations at this site. Second, Simon et al did not find PCLN-1 protein expression in human DCT [5].

Moreover, the present study provides additional information on the mechanism of the regulatory effect of basolateral calcium and magnesium concentration on their own reabsorption in the TAL, which is still debated. In a one patch-clamp study in rat TAL, on the basis of the observation that one apical K channel (70 pS) activity in TAL was inhibited by a high extracellular calcium concentration, the authors speculate that NaCl transport and lumen-positive transepithelial potential difference (PDte) might be suppressed, which in turn may be responsible for the inhibition of Mg and Ca paracellular reabsorption [23]. However, there is a growing body of evidence that TAL calcium and magnesium transport are regulated through changes in the paracellular pathway permeability without an alteration in the NaCl transport and PDte. Quamme and Dirks, in experiments using in vivo microperfusion of Henle's loop, showed that acute and selective increase in plasma Mg concentration in TPTX rats inhibits divalent cation, but not NaCl absorption [15]. Additionally, in rat and mouse TAL isolated in vitro it has been shown that a selective increase in bath calcium concentration impairs Ca and Mg reabsorption, but has no effect on PDte, with either no change (in mouse) [4] or a borderline reduction (in rat) in NaCl transport [24]. These data clearly suggest that increasing extracellular calcium or magnesium concentration inhibits their own reabsorption by a selective effect on the paracellular pathway. Our present study supports such a similar selective effect in humans. Indeed, in CS, as well as in ERH patient, acute magnesium infusion alone does not exert any natriuretic effect. In addition, acute hypermagnesemia in CS greatly reduces the calciuric and magnesuric effects of furosemide infusion but does not alter the natriuretic response to furosemide. Altogether, these data suggest that in humans, there is a selective inhibitory effect of high basolateral Mg^{2+}/Ca^{2+} concentration on TAL paracellular divalent cation reabsorption, as previously shown in animal models [4].

In conclusion, our study shows that the loss of function mutations in paracellin-1 results in a selective alteration in TAL paracellular Mg and Ca permeability, which is responsible for the selective defect in Mg and Ca renal reabsorption present in patients with HHS. In addition, these data support the theory of a selective regulatory effect of basolateral Ca^{2+} and Mg^{2+} concentrations on their own paracellular permeability in TAL. Because PCLN-1 bridges the paracellular junctions, it is in contact with both the intracellular and extracellular fluid. Thus, the intracellular domain possibly regulates the divalent cation paracellular pathway through the basolateral Ca/Mg-sensing receptor via intracellular messengers [4]. In addition, the high density of negative charges of the first extracellular domain of PCLN-1, which likely contributes to the cationic selectivity of the paracellular pathway, could be a divalent cation sensor [5]. Further studies are required to determine the precise cellular and molecular mechanisms of extracellular divalent cation concentration on paracellular PCLN-1 activity in the TAL.

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Reprint requests to Anne Blanchard, M.D., Département de Physiologie et Radio-Isotopes, Hôpital Européen Georges Pompidou, 20 rue Leblanc, 75908 Paris cédex 15, France.
E-mail: blanch@ccr.jussieu.fr

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