

## CaSR-mediated interactions between calcium and magnesium homeostasis in mice

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<sup>1</sup>Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital, Boston, Massachusetts; <sup>2</sup>Amgen Incorporated, Thousand Oaks, California; <sup>3</sup>Calcium Research Laboratory, Department of Medicine, McGill University, Montreal, Quebec, Canada; and <sup>4</sup>Renal Division, Beth Israel-Deaconess Medical Center, Boston, Massachusetts

Submitted 6 November 2012; accepted in final form 23 January 2013

**Quinn SJ, Thomsen AR, Egbuna O, Pang J, Baxi K, Goltzman D, Pollak M, Brown EM.** CaSR-mediated interactions between calcium and magnesium homeostasis in mice. *Am J Physiol Endocrinol Metab* 304: E724–E733, 2013. First published January 29, 2013; doi:10.1152/ajpendo.00557.2012.—Calcium (Ca) and magnesium (Mg) homeostasis are interrelated and share common regulatory hormones, including parathyroid hormone (PTH) and vitamin D. However, the role of the calcium-sensing receptor (CaSR) in Mg homeostasis in vivo is not well understood. We sought to investigate the interactions between Mg and Ca homeostasis using genetic mouse models with targeted inactivation of PTH (PTH KO) or both PTH and the calcium-sensing receptor (CaSR) (double knockout, DKO). Serum Mg is lower in PTH KO and DKO mice than in WT mice on standard chow, whereas supplemental dietary Ca leads to equivalent Mg levels for all three genotypes. Mg loading increases serum Mg in all genotypes; however, the increase in serum Mg is most pronounced in the DKO mice. Serum Ca is increased with Mg loading in the PTH KO and DKO mice but not in the WT mice. Here, too, the hypercalcemia is much greater in the DKO mice. Serum and especially urinary phosphate are reduced during Mg loading, which is likely due to intestinal chelation of phosphate by Mg. Mg loading decreases serum PTH in WT mice and increases serum calcitonin in both WT and PTH KO mice but not DKO mice. Furthermore, Mg loading elevates serum 1,25-dihydroxyvitamin D in all genotypes, with greater effects in PTH KO and DKO mice, possibly due to reduced levels of serum phosphorus and FGF23. These hormonal responses to Mg loading and the CaSR's role in regulating renal function may help to explain changes in serum Mg and Ca found during Mg loading.

calcium-sensing receptor; calcium; magnesium; parathyroid hormone; 1,25-dihydroxyvitamin D<sub>3</sub>; calcitonin; osteocalcin; alkaline phosphatase; deoxyypyridinoline

MAGNESIUM (Mg) is an essential divalent cation that has a plethora of roles in the body, particularly intracellularly, where it participates in energy metabolism and intracellular signaling through its involvement in multiple phosphorylation reactions. Not surprisingly, the level of extracellular Mg is maintained within a relatively narrow range of ~0.75–1.05 mmol/l. This is slightly lower than the level at which extracellular calcium (Ca) is maintained (serum ionized Ca = ~1.1–1.3 mmol/l). Extracellular Ca homeostasis comprises three key elements: 1) tissues that transport Ca into or out of the extracellular fluid (kidney, bone, and intestine), 2) hormones that regulate these fluxes {e.g., parathyroid hormone (PTH), 1,25-dihydroxyvita-

min D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], calcitonin (CT), and fibroblast growth factor 23 (FGF23)}, and 3) one or more Ca sensors [viz., the calcium-sensing receptor (CaSR)] that measure the level of Ca in the blood and regulate the secretion of Ca-regulating hormones or Ca fluxes themselves (e.g., in the kidney) (3).

Less is known about the mechanisms underlying the maintenance of Mg homeostasis than of Ca homeostasis, although there have been recent important advances in the former, including the discovery of Mg channels, particularly transient receptor potential cation channel subfamily M member 6 (TRPM6), that participate in Mg absorption by the gastrointestinal tract and reabsorption in the kidney (28–30). 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to stimulate intestinal absorption of Mg in some but not other studies, and so it may participate in ensuring the availability of sufficient Mg to provide for the body's needs (10, 23). In addition, PTH stimulates renal tubular reabsorption of Mg in some species, especially the golden hamster (11). Even without PTH, peritubular (but not luminal) Mg as well as Ca inhibit the reabsorption of both Mg and Ca in the thick ascending limb (TAL) of Henle's loop in the kidney (5). This "autoregulation" of tubular reabsorption of Mg in the TAL is thought to be a key factor contributing to Mg homeostasis.

The identity of the putative Mg sensor(s) regulating PTH secretion and renal tubular reabsorption of Mg has been elusive. Genetic evidence has suggested that the CaSR may also serve not only as a Ca sensor but also as a Mg sensor in vivo, since inactivating mutations of this receptor are associated with high/normal or mildly elevated levels of serum Mg, whereas conversely there can be hypomagnesemia in patients with activating mutations of the receptor (12, 18, 21, 22). However, whether Mg regulates the CaSR and the CaSR participates in Mg homeostasis in vivo has been uncertain. For example, the levels of Mg activating the CaSR are generally about twice as high as the concentrations of Ca stimulating the receptor and are seemingly too high to be achieved in vivo under normal physiological conditions (3). For example, only at supraphysiological levels, such as those occurring during treatment of preeclampsia with Mg infusion, does Mg inhibit PTH secretion substantially in humans, and it is unclear whether the actions of normal levels of Mg contribute on a minute-to-minute basis to the CaSR-mediated control of PTH secretion (7, 9). This form of Mg therapy also promotes marked hypercalciuria and magnesuria potentially via the associated inhibition of PTH secretion and/or a direct effect of hypermagnesemia on the TAL (7, 9). However, it has not been formally proven that this stimulation of Mg and Ca excretion is mediated by the CaSR.

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The goal of the present study was to utilize mice with targeted deletion ["knockout" (KO)] of PTH (PTH KO), of both PTH and the CaSR [double knockout (DKO)], or of neither [wild type (WT)] to investigate the roles of PTH and the CaSR in the defense against oral Mg loading. The rationale behind the use of this model is described in detail in MATERIALS AND METHODS. By measuring changes in the levels of both Mg and Ca in serum and urine as well as the levels of hormones potentially participating in Mg homeostasis [e.g., 1,25(OH)<sub>2</sub>D<sub>3</sub> and CT], we have demonstrated altered regulation of not only Mg but also Ca during Mg loading that results from loss of PTH or of both PTH and the CaSR. These results indicate that PTH and the CaSR, particularly the latter, are important in the defense against oral Mg loading and that associated changes in Ca metabolism result, at least in part, from phosphate (P<sub>i</sub>) depletion, presumably owing to chelation of P<sub>i</sub> by Mg in the gut and resultant increases in 1,25(OH)<sub>2</sub>D<sub>3</sub>.

## MATERIALS AND METHODS

**Generation of PTH KO and DKO mouse lines.** The two parental strains of CaSR<sup>-/-</sup> mice and PTH<sup>-/-</sup> mice were generated by homologous recombination in embryonic stem cells (12, 16). Mice heterozygous for KO of exon 5 of the *Casr* gene as well as mice heterozygous for the null *Pth* allele are fertile and were bred to generate offspring heterozygous at both the *Casr* and *Pth* loci. The latter were then used to generate CaSR<sup>+/+</sup>/PTH<sup>+/+</sup> (referred to as WT), CaSR<sup>+/+</sup>/PTH<sup>-/-</sup> (PTH KO), and CaSR<sup>-/-</sup>/PTH<sup>-/-</sup> (DKO) mice. We maintained lines of DKO, PTH KO, and WT mice that had been back-crossed extensively on a mixed genetic background comprising contributions from C57B6, 129/svJ, and 129/SvEv strains. These three lines were periodically back-crossed to minimize genetic drift, and biochemical parameters in blood and urine were tested periodically to ensure stability of the biochemical phenotypes.

**Rationale behind the use of the PTH KO and DKO mouse models.** The neonatal lethality of the homozygous CaSR KO mice has limited their utility for studying the physiological roles of the CaSR *in vivo*. Of the other mouse models available for such studies, one "rescues" homozygous CaSR KO mice by crossing heterozygous CaSR KO mice with mice with KO of the PTH gene. This model has been utilized to show that the CaSR is a key contributor to the fine control of the serum Ca concentration (14) and the defense against Ca loads (8, 13). This rescued mouse model was generated utilizing the original mouse KO model in which exon 5 of the *Casr* gene was disrupted by inserting the neomycin gene (14). However, subsequently, it has become clear that an alternatively spliced CaSR lacking exon 5 can be produced in some tissues in homozygous CaSR KO mice with this genotype (19, 20). It has not yet been possible to express high enough levels of this shorter form of the receptor on the cell surface of heterologous cell systems to directly document that it actually has biological activity. However, chondrocytes from exon 5 CaSR KO mice still respond to Ca, providing indirect evidence that the CaSR lacking exon 5 may have biological activity in some tissues, especially bone and cartilage (26). Nevertheless, mice with homozygous global exon 5 KO of the CaSR clearly have severely deranged Ca-regulated PTH release and parathyroid cellular proliferation, causing death shortly after birth (14). In addition, we have shown previously that the DKO mice show nearly complete loss of high oral Ca-stimulated CT secretion and a striking inability to increase renal Ca excretion during marked hypercalcemia induced by a PTH infusion (13). An important advantage of the rescued global exon 5 KO mice is that they allow studies of the manner in which various CaSR-regulated tissues participating in Ca homeostasis interact and contribute to the observed derangements in Ca metabolism. In effect, normal homeostatic loops that are needed to maintain a stable level of Ca, including regulation

of PTH secretion, CT secretion, and perhaps 1,25(OH)<sub>2</sub>D<sub>3</sub> production by the CaSR, have been interrupted, providing a model in which these hormones can be added back in a controlled manner if desired.

**Genotyping of mice.** During generation of the three genotypes of experimental mice from the double-heterozygous mice, CaSR<sup>+/-</sup>/PTH<sup>+/-</sup>, genomic DNA was isolated using a kit (DNeasy kit; Qiagen, Valencia, CA). To determine the genotype at both the *Pth* and *Casr* loci (owing to their common usage in the literature, the proteins corresponding to these genes are designated here as PTH and CaSR), four PCR amplifications were carried out for each animal. To document the presence of the WT *Casr* allele, DNA samples were amplified with a *Casr* forward primer (5'-TCATTGATGAAC-AGTCTTTCTCCCT-3') and a *Casr* reverse primer (5'-TCTGT-TCTCTTTAGGTCCTGAAACA-3'). To document the presence of the KO *Casr* allele, a Neo forward primer (5'-TCTTGATTC-CCACTTTGTGGTTCTA-3') was utilized with the *Casr* reverse primer (5'-TCATTGATGAACAGTCTTTCTCCCT-3'). The WT *Pth* allele was detected using a *Pth* forward primer (5'-GATGTCTG-CAAACACCGTGGCTAA-3') and a *Pth* reverse primer (TCCA-AAGTTTCATTACAGTAGAAG). The null *Pth* allele was documented using the neo forward primer (5'-TCTTGATTC-CCACTTTGTGGTTCTA-3') and the *Pth* reverse primer (5'-TCCAAAGT-TTCATTACAGTAGAAG-3'). All PCR reactions were performed using "hot start" PCR with the following temperature cycling protocol: 94°C for 3 min, 94°C for 45 s, 56°C for 30 s, and 72°C for 1 min for 35 cycles (8, 13).

***In vivo* experiments.** The study was carried out using 3- to 9-mo-old male mice. Animal protocols were approved by the Institutional Animal Care and Use Committee at Harvard Medical School and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were housed in microisolator cages in a pathogen-free facility according to the regulations of the Harvard Medical School Center for Animal Resources and Comparative Medicine. As described below (see RESULTS), in some experiments PTH KO and DKO mice were hypocalcemic (owing to their hypoparathyroidism) when fed standard mouse chow (with 0.8% Ca wt/wt and 0.4% P<sub>i</sub>, Harlan Teklad-TD99224; Harlan-Teklad, Madison, WI) as well as drinking water with no added Ca (referred to as 0% Ca water). In other experiments, the three genotypes were rendered equivalently normocalcemic by feeding the DKO and PTH KO mice the same chow combined with drinking water supplemented with CaCl<sub>2</sub> [0.75 (wt/vol) and 1.5% (wt/vol), respectively]. The percentage of Ca expressed as wt/vol refers to the weight of the CaCl<sub>2</sub> salt added to the water and not to elemental Ca. Mice were subjected to various experimental manipulations, as described in RESULTS, and blood and urine samples were collected for determination of the analytes described below.

**Blood and urine measurements.** Serum and urine analytes were measured using the following kits: serum and urinary Ca and Mg (Eagle Diagnostics, De Soto, TX); serum and urinary creatinine (Cr), serum and urinary phosphorus, and alkaline phosphatase (Stanbio Laboratory, Boerne, TX); 1,25(OH)<sub>2</sub>D<sub>3</sub> by RIA (Immunodiagnosics Systems, Fountain Hills, AZ); urinary deoxyypyridinoline (DPD) cross-links (Quidel, San Diego, CA); osteocalcin, PTH, and CT (Alpco Diagnostics, Salem, NH); and genotyping, DNeasy kit and primers (Qiagen).

**Statistics.** Statistical analyses were carried out by unpaired *t*-test when two groups were compared or by ANOVA using Dunnett's *t*-test for comparisons of three or more group means. Statistical calculations used an *n* equal to the number of mice in each group; a *P* value of <0.05 was taken to indicate statistical significance. Statistical comparisons of PTH KO and DKO mice were made with their respective genetic controls, WT and PTH KO mice, since the comparison of DKO and WT mice is across two genetic changes (e.g., loss of both PTH and the CaSR), complicating interpretation of any observed differences. In general, each experiment was performed at

least twice with eight mice of each genotype in each experiment. Results were pooled if the experimental design was identical.

## RESULTS

*Effects of Mg loading on serum and urinary Mg levels in WT, PTH KO, and DKO mice.* To assess the effects of Mg loading, all three genotypes of mice were kept on a normal chow diet either with or without 100 mM Mg in their drinking water. Mice drank equivalent amounts (3–4 ml) with or without Mg in the water. Figure 1, A and B, shows serum and urinary Mg concentrations when the mice were drinking plain water or water containing 100 mM MgCl<sub>2</sub> for 7 days. On a normal diet, the serum Mg concentrations of the PTH KO and DKO mice were reduced to an equivalent extent compared with WT mice, indicating that the change in serum Mg was a phenotype resulting from PTH deficiency and was not further impacted by loss of the full-length CaSR (Fig. 1A). During Mg loading, serum Mg increased in all three genotypes to essentially the same level (~3 meq/l); however, the increment above baseline observed in the PTH KO and DKO mice during Mg

loading was greater than that observed in the WT mice (Fig. 1A). Under baseline conditions, spot urinary Mg, expressed as mg and normalized to mg Cr, varied from ~1 to 1.7 mg/mg in the three genotypes, with the DKO mice showing a lower urinary Mg than the other genotypes (Fig. 1B). The somewhat higher baseline urinary Mg/Cr in the PTH KO mice than in the WT mice, although not statistically significant, could have contributed to the lower level of serum Mg in the former. Clearly, however, urinary Mg wasting could not explain the reduction in serum Mg observed in the DKO mice, since their urinary Mg/Cr was, if anything, lower than in the other two genotypes.

As shown in our previous studies (13), supplementing the water of the PTH KO and DKO mice with 1.5 and 0.75% CaCl<sub>2</sub>, respectively, increased their serum Ca concentrations to levels equivalent to that of the WT mice. Therefore, to test the effects of Mg loading on the three genotypes when their levels of serum Ca were equivalent, serum and urinary Mg concentrations were measured after 1 wk of Ca supplementation in the two KO genotypes with or without Mg loading as before

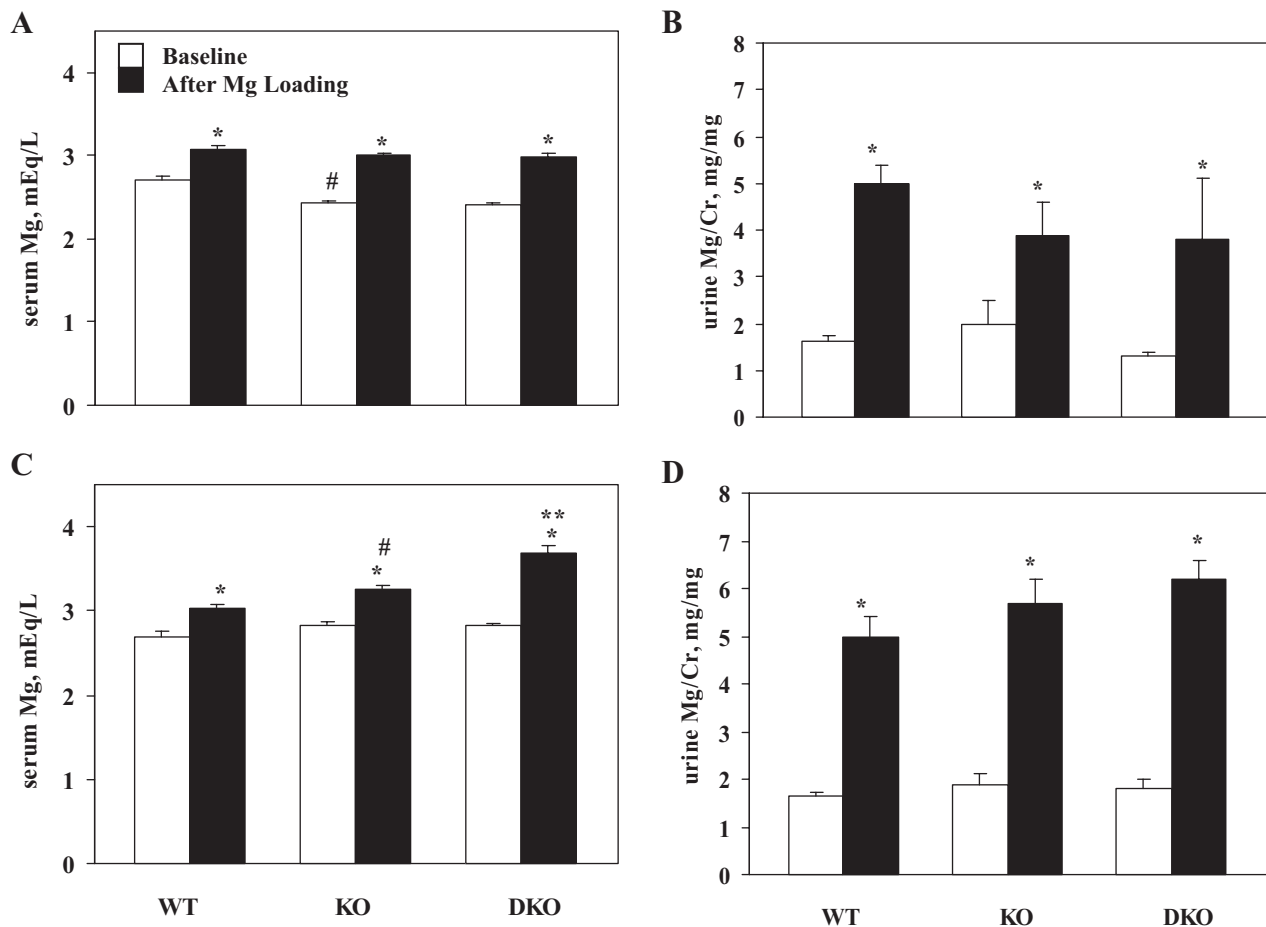


Fig. 1. A: effect of 7 days of magnesium (Mg) supplementation on serum Mg in mice receiving plain drinking water. B: effect of 7 days of Mg supplementation on urinary Mg normalized to urinary creatinine (Cr) in mice receiving plain drinking water. C: effect of 7 days of Mg supplementation on serum Mg in wild-type (WT) mice receiving plain water as well as in parathyroid hormone (PTH) knockout (KO) and double knockout (DKO) mice receiving 1.5 and 0.75% calcium (Ca), respectively, in their drinking water. D: effect of 7 days of Mg supplementation on urinary Mg/Cr in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values. In this and subsequent figures, statistical comparisons of PTH KO and DKO mice with their respective genetic controls, WT and PTH KO mice, were made since comparison of DKO with WT mice is across 2 genetic changes (e.g., loss of both PTH and the CaSR), complicating the interpretation of any observed differences.



(Fig. 1, C and D). Note that addition of Ca to the drinking water substantially increased baseline serum Mg concentrations in the PTH KO and DKO mice by  $\sim 0.4$  meq/l, resulting in similar serum Mg concentrations in the three genotypes. As before, Mg loading increased serum Mg by  $\sim 0.25$  meq/l in the WT mice. A greater increase in serum Mg was observed in the PTH KO mice and DKO mice, with each being significantly greater than its genetic control. Figure 1D shows the changes in urinary Mg/Cr in this experiment. At baseline, the mice had similar levels of urinary Mg/Cr of  $\sim 1.5$  mg/mg, and all three genotypes increased urinary Mg/Cr about threefold (Fig. 1D).

**Effects of Mg loading on serum and urinary Ca levels in WT, PTH KO, and DKO mice.** Since both Ca and Mg can bind to and activate the CaSR, we also measured serum and urinary Ca in the experimental paradigm just described to determine whether there were associated changes in Ca homeostasis. Figure 2A shows that, as expected, the two PTH-deficient genotypes (PTH KO and DKO) were hypocalcemic ( $\sim 7$  mg/dl), whereas the WT mice were normocalcemic ( $\sim 10.4$  mg/dl) when there was no Ca supplementation of the drinking water. Of interest, Mg loading had no effect on serum Ca in the WT mice but substantially increased serum Ca in the two KO genotypes to levels of  $\sim 8.5$  and  $9$  mg/dl in the PTH KO and DKO mice, respectively. Figure 2B shows that Mg loading

increased urinary Ca/Cr to similar extents (3- to 4-fold) in all three genotypes from 0.2 to 0.4 mg/mg to  $\sim 1.5$  to  $1.9$  mg/mg. Therefore, the much greater increase in serum Ca concentration with Mg loading in the two KO genotypes than in the WT mice cannot be explained by reduced renal excretion of Ca.

Figure 2C shows serum Ca in the three genotypes with or without Mg loading for 7 days, when the two KO genotypes were maintained normocalcemic by Ca supplementation, as described above. Prior to Mg loading, the three genotypes had similar values of serum Ca, ranging from 10.1 to 10.6 mg/dl. Mg loading produced an increase in serum Ca in all three genotypes, with significantly greater increases in serum Ca concentration found in both the PTH KO and the DKO mice compared with the WT mice. This increase was particularly striking for the DKO mice, which exhibited severe hypercalcemia following Mg loading. Figure 2D shows the marked increase in urinary Ca/Cr in the PTH KO mice receiving 0.75% Ca in their drinking water, reflecting the marked renal Ca "leak" resulting from lack of PTH and, presumably, activation of the CaSR in the TAL as serum Ca increased into the midnormal range or higher. The DKO mice had a fourfold lower urinary Ca/Cr under baseline conditions, likely owing to their inability to upregulate urinary Ca excretion to the same extent as the PTH KO mice. The WT and DKO mice showed

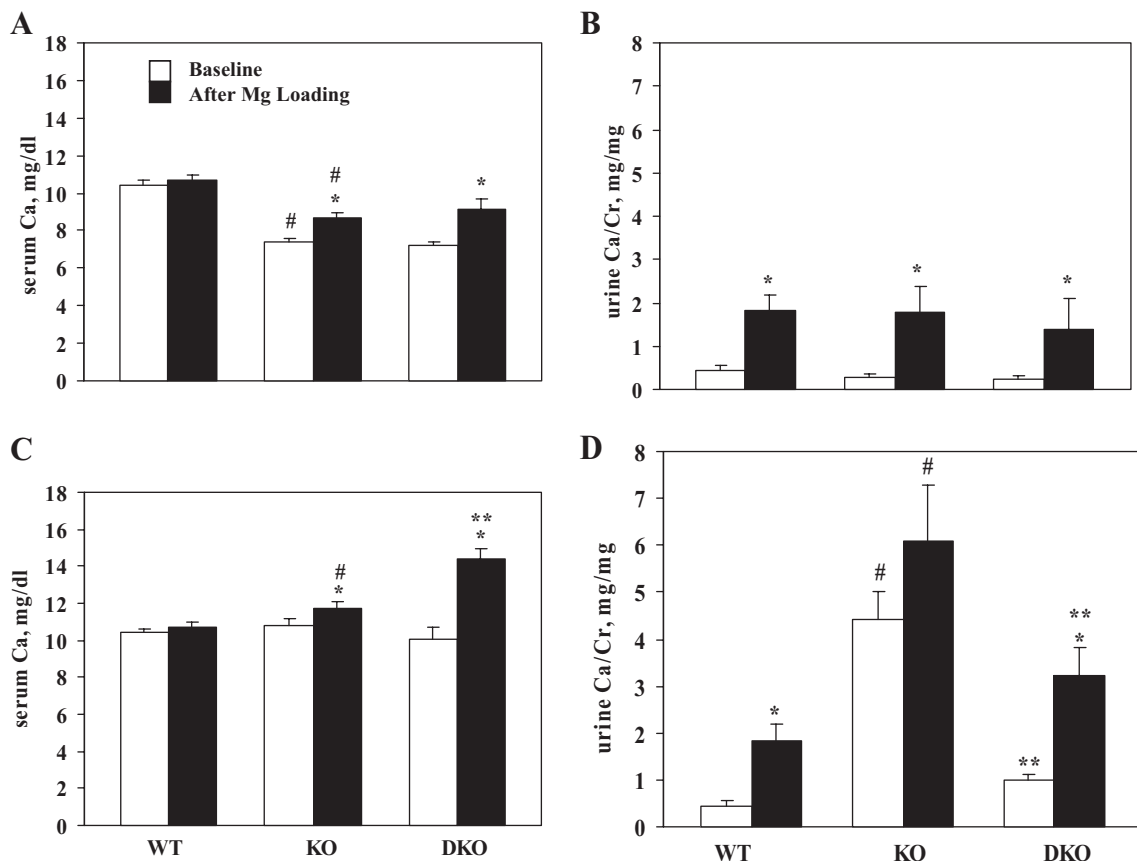


Fig. 2. A: effect of 7 days of Mg supplementation on serum Ca in mice receiving plain drinking water. B: effect of 7 days of Mg supplementation on urinary Ca/Cr in mice receiving plain drinking water. C: effect of 7 days of Mg supplementation on serum Ca in WT mice receiving plain water as well as in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. D: effect of 7 days of Mg supplementation on urinary Ca/Cr in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\*\* $P < 0.05$  for comparison of PTH KO to DKO values.

significant fold increases in urinary Ca/Cr during Mg loading, which were similar in magnitude. The difference in renal Ca handling between the PTH KO and DKO mice was also striking during Mg loading, when the absolute level of urinary Ca/Cr in the DKO mice was one-half that of the PTH KO mice despite the marked hypercalcemia in the DKO mice.

**Effect of Mg loading on serum and urinary  $P_i$  in WT, PTH KO, and DKO mice.** Figure 3A shows the expected elevations in serum  $P_i$  in the hypoparathyroid PTH KO and DKO mice relative to that in the WT mice under baseline conditions, owing to the lack of the phosphaturic action of PTH in the two KO genotypes. Mg loading reduced serum  $P_i$  modestly in the WT mice (by  $\sim 1$  mg/dl) and by  $\sim 2$  mg/dl in the PTH KO and DKO mice. This suggested the possibility of chelation of dietary  $P_i$  in the gut by the addition of Mg in the drinking water. Likewise, urinary  $P_i$ /Cr was markedly decreased (5- to 10-fold) in all three genotypes during Mg loading, presumably owing to reduced gastrointestinal availability and absorption of  $P_i$  (Fig. 3B).

Figure 3, C and D, shows that Mg loading also reduced serum  $P_i$  and urinary  $P_i$ /Cr when the PTH KO and DKO mice were maintained normocalcemic with Ca supplementation. These results differ from those in Fig. 3, A and B, in that urinary  $P_i$ /Cr was also low in the PTH KO and DKO mice under baseline conditions, presumably because of chelation of  $P_i$  in the gut by the supplemental Ca in the water. Despite the

very low urinary  $P_i$ /Cr in the PTH KO and DKO mice, the serum  $P_i$  levels remained high, particularly in the DKO mice at baseline (Fig. 3C). However, with Mg loading, overtly reduced serum  $P_i$  levels, which were lower than those observed during Ca supplementation alone, were observed in the PTH KO and DKO mice, suggesting profoundly deficient  $P_i$  absorption (Fig. 3C).

**Effect of Mg loading on bone turnover.** Because Mg loading might affect Ca and Mg homeostasis by changing bone turnover, we measured markers of both bone turnover and resorption. Figure 4 shows that the PTH KO mice had a level of alkaline phosphatase under baseline conditions that was significantly lower than that of the WT mice, perhaps reflecting the lack of PTH in the PTH KO mice. Mg loading did not have any consistent effect on serum alkaline phosphatase under baseline or Mg-loaded conditions regardless of whether the PTH KO and DKO mice were hypo- or normocalcemic (Fig. 4).

Similar results were observed for serum osteocalcin. Figure 5 shows that baseline serum osteocalcin concentrations were lower in the two KO genotypes regardless of whether they were hypo- or normocalcemic. Mg loading had little or no effect on serum osteocalcin in the three genotypes, although it increased osteocalcin modestly in the DKO mice when they were supplemented with Ca.

Figure 6 illustrates that baseline DPD levels were lower in the PTH KO and DKO mice than in the WT mice regardless of whether they were hypo- or normocalcemic at baseline. Mg

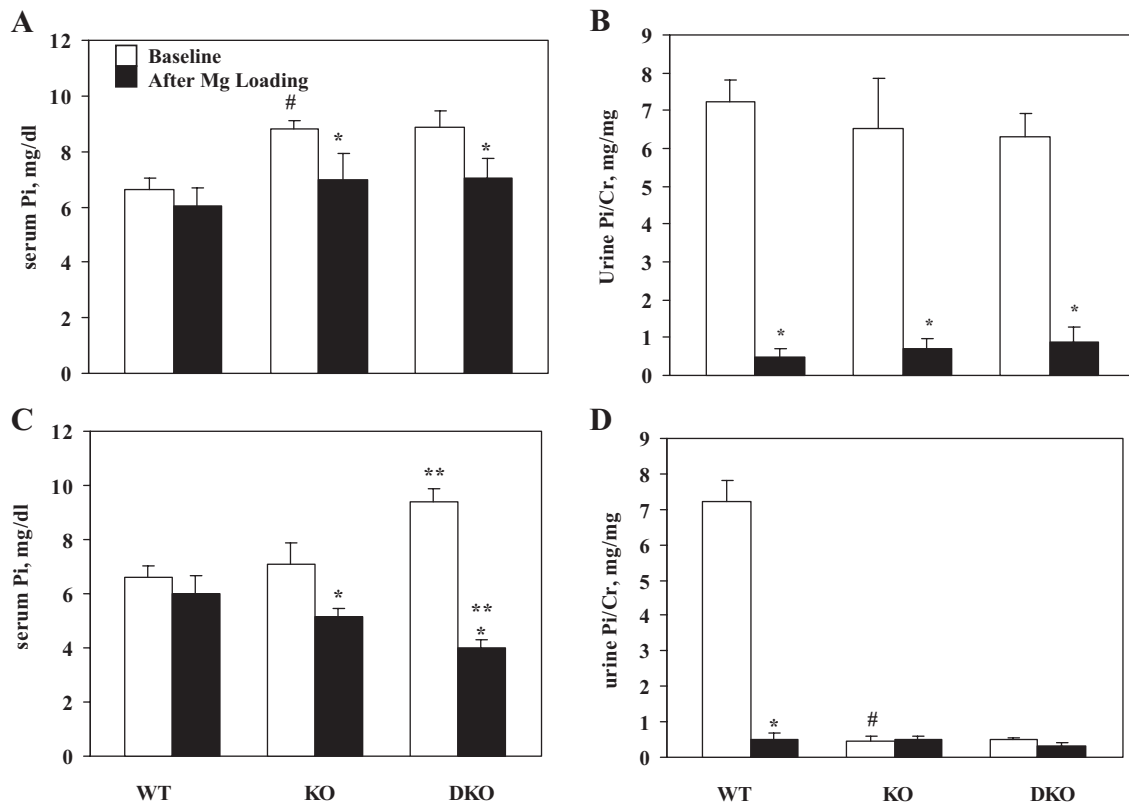


Fig. 3. A: effect of 7 days of Mg supplementation on serum  $P_i$  in mice receiving plain drinking water. B: effect of 7 days of Mg supplementation on urinary  $P_i$ /Cr in mice receiving plain drinking water. C: effect of 7 days of Mg supplementation on serum  $P_i$  in WT mice receiving plain water as well as in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. D: effect of 7 days of Mg supplementation on urinary  $P_i$ /Cr in WT mice receiving plain water or in PTH KO and DKO mice supplemented with 1.5 and 0.75% Ca, respectively. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

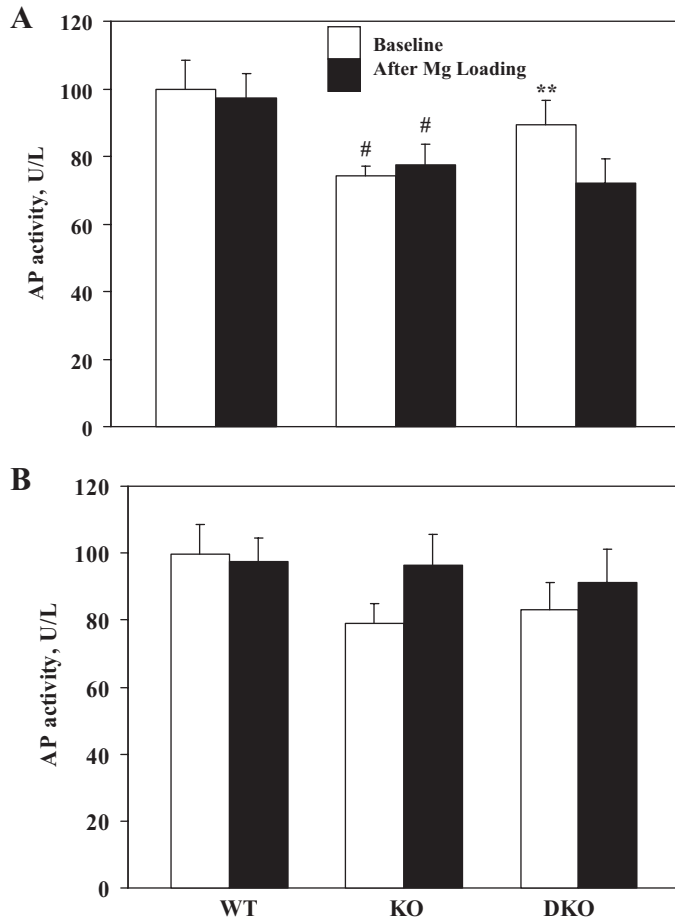


Fig. 4. *A*: effects of Mg loading on serum alkaline phosphatase (AP) concentration in WT, PTH KO, and DKO mice receiving plain drinking water. *B*: effect of 7 days of Mg supplementation on serum AP in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

loading had no effect on DPD in the WT mice, but when the PTH KO and DKO mice were loaded with Mg during Ca supplementation (with resultant normocalcemia), their serum DPD concentrations increased by  $\sim 40$ – $50\%$ .

**Effect of Mg loading on serum CT.** Figure 7*A* shows that Mg loading produced about a twofold but statistically insignificant increase in serum CT in the normocalcemic WT mice but had no effect on either the PTH KO or DKO mice. However, when the PTH KO and DKO mice were maintained normocalcemic under baseline conditions by supplementation of their drinking water with Ca, the PTH KO mice showed a substantial increase in CT under baseline conditions and a marked increase during Mg supplementation (Fig. 7*B*). In contrast, the DKO mice showed only a modest increase in serum CT concentration despite their marked elevation in serum Ca concentration during Mg loading, confirming our prior demonstration of loss of stimulation of CT by Ca in this genotype (13).

**Effect of Mg loading on serum  $1,25(\text{OH})_2\text{D}_3$  and PTH.** Figure 8*A* shows that Mg loading increased serum  $1,25(\text{OH})_2\text{D}_3$  levels in all three genotypes when they received plain water, although the magnitude of the increment in the KO genotypes

was about three to four times greater than that found in the WT mice. A similar pattern was observed when the PTH KO and DKO mice were maintained normocalcemic by Ca supplementation (Figure 8*B*), although the  $1,25(\text{OH})_2\text{D}_3$  level in the PTH KO mice was increased even further compared with those of the other two genotypes. As expected, Mg loading of WT mice reduced serum PTH by 30–40% ( $P < 0.05$ ; Fig. 9). Similar studies were not performed in the other two genotypes given their hypoparathyroid state.

## DISCUSSION

The results of this study provide insights into the system maintaining Mg homeostasis as well as the relationships between this system and those maintaining Ca and  $\text{P}_i$  homeostasis. Based on our earlier studies of the role of the CaSR in the defense against hypercalcemia induced by Ca loading,  $\text{P}_i$  depletion, or  $1,25(\text{OH})_2\text{D}_3$  administration and the known activation of the CaSR by Mg, we anticipated that KO of the CaSR would also impact Mg homeostasis (4, 8, 13). We chose to utilize Mg loading to carry out these studies, as Mg depletion has paradoxical effects on the CaSR and on PTH secretion (1, 25). We used the DKO mice, since the role of the CaSR in Ca homeostasis was particularly apparent in these mice in terms of their impaired defense against hypercalcemia and, potentially, hypermagnesemia.

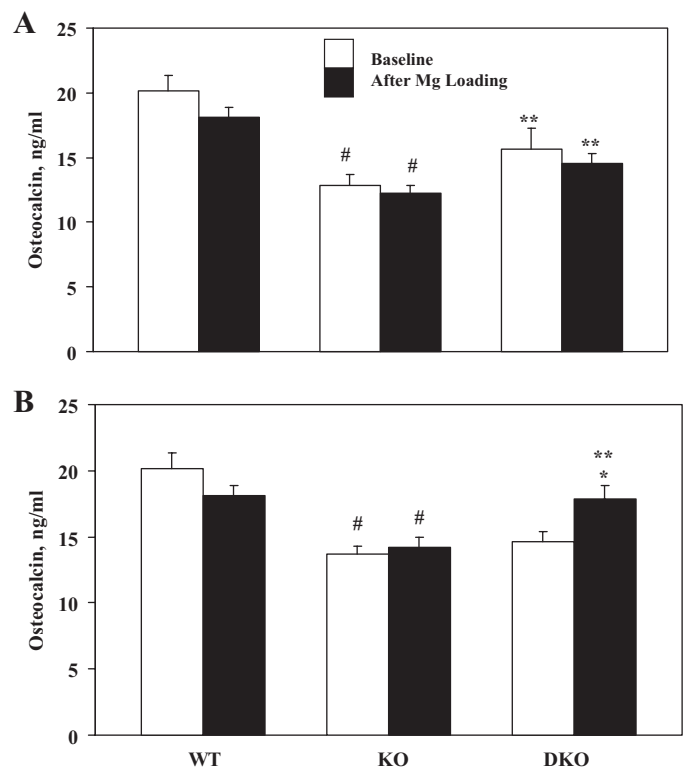


Fig. 5. *A*: effects of Mg loading on osteocalcin concentration in WT, PTH KO, and DKO mice receiving plain drinking water. *B*: effect of 7 days of Mg supplementation on serum osteocalcin in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

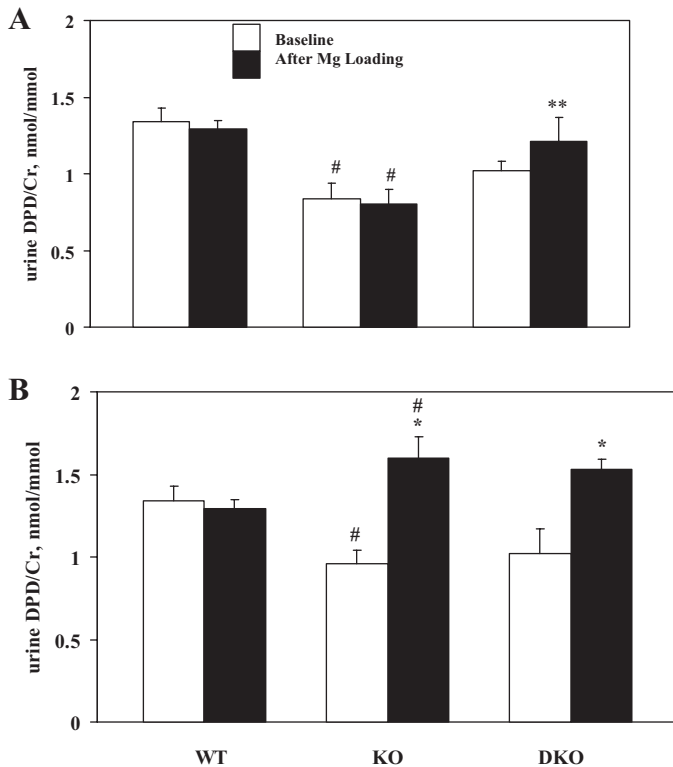


Fig. 6. *A*: effects of Mg loading on urinary deoxyypyridinoline (DPD)/Cr in WT, PTH KO, and DKO mice receiving plain drinking water. *B*: effect of 7 days of Mg supplementation on urinary DPD/Cr in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

At baseline, with dietary Ca (0.8%) present only in the chow, hypoparathyroid PTH KO and DKO mice had modestly but statistically significantly lower serum Mg concentrations (by  $\sim 10\%$ ) than the WT mice. Frank hypomagnesemia can be present in acquired hypoparathyroidism as well as in patients with activating mutations of the CaSR, who have not only functional hypoparathyroidism but also an activated CaSR in the renal tubule that is thought to promote renal Ca excretion (2, 8, 18). This reduction in serum Mg in the PTH KO and DKO mice represents a phenotype of PTH and not CaSR deficiency in our mouse model, since the PTH KO and DKO mice had comparable reductions in serum Mg.

When 100 mM Mg was added to the drinking water of the three genotypes when they received normal chow without additional Ca supplementation, all exhibited an increase in serum Mg concentration to a similar level ( $\sim 3$  meq/l), although the increment from baseline was twice as great for the PTH KO and DKO mice as for the WT mice, with the two KO genotypes showing similar increases (Fig. 1A). Therefore, because the DKO mice underwent a substantially greater increase in serum Ca than the PTH KO mice when their drinking water was supplemented with any given concentration of Ca, the CaSR is apparently not as important in defending against Mg loading as it is in protecting against Ca loading under these experimental conditions (13). In fact, all three genotypes exhibited a two- to threefold increase in urinary Mg concentration

during Mg loading, and urinary Mg concentration was equivalent in the PTH KO and DKO mice (Fig. 1B). Of note, however, is that when serum Ca levels in the PTH KO and DKO mice were maintained normal by adding Ca to the drinking water, Mg loading produced a substantially greater increase in serum Mg in the DKO mice than in the PTH KO mice (Fig. 1C), perhaps because there is a limited capacity of the DKO mice to excrete a Mg load in the setting of a Ca load in the presence of CaSR deficiency.

Mg loading impacts not only Mg but also Ca homeostasis. Unlike WT mice, PTH KO and DKO mice exhibited significant increases in serum Ca during Mg loading to nearly normal levels in the absence of Ca in the water and with a constant Ca content in the chow (Fig. 2A). These increases in the KO genotypes could not be explained by reduced Ca excretion because urinary Ca levels were equivalent in all three genotypes (Fig. 2B). The changes in serum Ca were even more marked during Ca supplementation, especially in the DKO

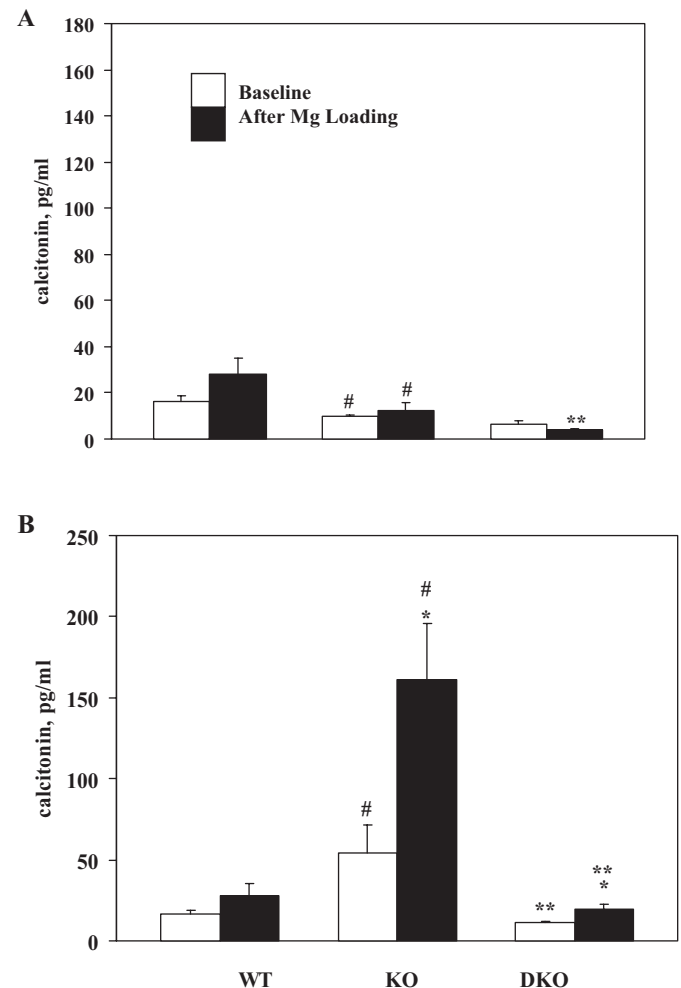


Fig. 7. *A*: effects of Mg loading on serum calcitonin concentration in WT, PTH KO, and DKO mice receiving plain drinking water. *B*: effect of 7 days of Mg supplementation on serum calcitonin in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

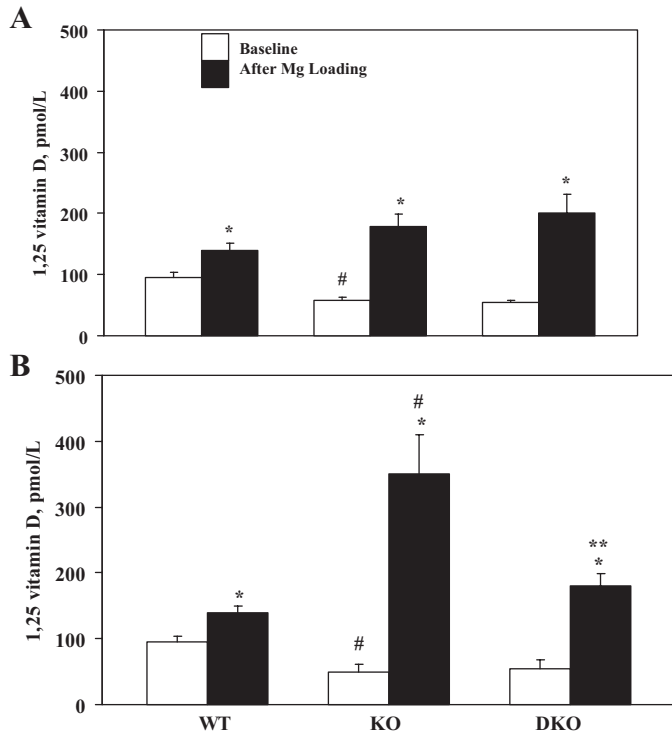


Fig. 8. *A*: effects of Mg loading on serum 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] concentration in WT, PTH KO, and DKO mice receiving plain drinking water. *B*: effect of 7 days of Mg supplementation on serum 1,25(OH)<sub>2</sub>D<sub>3</sub> in WT mice receiving plain water or in PTH KO and DKO mice receiving 1.5 and 0.75% Ca, respectively, in their drinking water. Values are means  $\pm$  SE ( $n = 8$ /genotype). \* $P < 0.05$  for comparison of Mg-loaded values with the respective control values; # $P < 0.05$  for comparison of WT with PTH KO values; \*\* $P < 0.05$  for comparison of PTH KO with DKO values.

mice, whose serum Ca increased from a normal level at baseline to marked hypercalcemia ( $\sim 15$  mg/dl) during Mg loading (Fig. 2C). Urinary Ca/Cr at baseline in the PTH KO mice was markedly higher than the other two genotypes during Ca supplementation, reflecting the pronounced renal Ca “leak” observed in this genotype when given a Ca loading to maintain normocalcemia (Fig. 2D). This enhanced urinary Ca excretion in the PTH KO mice is presumably the result of the absence of PTH and some degree of activation of the CaSR in the TAL when serum Ca is maintained at a high/normal level by Ca supplementation. The difference in urinary Ca/Cr between the PTH KO mice and the other two genotypes persisted and was amplified during Mg loading. Urinary Ca/Cr in the DKO mice was only about one-half that in the PTH KO mice despite the markedly higher serum Ca in the former. In contrast to the Mg load-induced changes in urinary Mg observed in Ca-supplemented mice, loss of the full length CaSR in the DKO mice produced a relatively greater reduction in urinary Ca/Cr than in urinary Mg/Cr. This result suggests that in the mouse models used here the CaSR has a greater role in regulating renal Ca than renal Mg handling, although loss of the CaSR clearly impairs the capacity of the Ca-supplemented, normocalcemic DKO mice to mitigate the increase in serum Mg during Mg loading.

There was an increase in serum CT and a reduction in serum PTH in the WT mice during Mg loading (Figs. 7 and 9). The stimulation of a Ca-lowering hormone and inhibition of a

Ca-elevating hormone likely mitigated the changes in serum Ca and Mg observed in the WT mice during Mg loading. Mg, like Ca, is known to activate the CaSR and inhibit PTH release, but it is less clear whether Mg stimulates CT secretion (4, 7, 9, 15). Mg loading increased circulating levels of CT in the PTH KO mice, but only when they were made normocalcemic by Ca supplementation. It is likely that the hypocalcemia present in these mice when no Ca was added to their drinking water did not support stimulation of CT, whereas the mild hypercalcemia induced in the Ca-supplemented PTH KO mice enhanced CT release either by itself or through the combined effects of increases in both Ca and Mg on the CaSR. Despite the marked increases in both serum Ca and serum Mg in the Ca-supplemented DKO mice, CT did not rise above 20 pg/ml. Thus the data observed here with the DKO mice support our prior conclusion that the full-length CaSR is a critical mediator of the stimulatory effect of high Ca on CT secretion. The lack of the CT response during Mg loading could potentially limit the ability of DKO mice to reabsorb Mg in the urine, similar to that found with Ca, yet a more profound hypermagnesemia is seen in DKO vs. PTH KO mice.

Our studies of the effects of Mg loading on serum and urinary P<sub>i</sub> concentrations provided a clue as to why serum and urinary Ca concentrations increase during Mg loading in the three genotypes of mice in the absence of any change in dietary Ca load. There was a Mg loading-induced reduction in serum P<sub>i</sub> both when the mice were drinking plain water and when the PTH KO and DKO mice received Ca in their drinking water to maintain normocalcemia (Fig. 3). Although the changes in serum P<sub>i</sub> were relatively small, especially when all genotypes were receiving plain water, urinary P<sub>i</sub> was markedly reduced during Mg supplementation in both experiments, strongly suggesting that gastrointestinal absorption of P<sub>i</sub> was reduced and that the kidney sensed P<sub>i</sub> depletion. The P<sub>i</sub> depletion resulting from oral Mg supplementation is not surprising in view of the fact that the solubility products for CaHPO<sub>4</sub> and MgHPO<sub>4</sub> are similar (6). The P<sub>i</sub> depletion resulting from chelation of P<sub>i</sub> by Mg in the gastrointestinal tract would be expected to increase the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> through a direct effect of P<sub>i</sub> depletion on the 1 $\alpha$ -hydroxylase in the renal proximal tubule or a decline in FGF23 release by osteocytes, a potent tonic

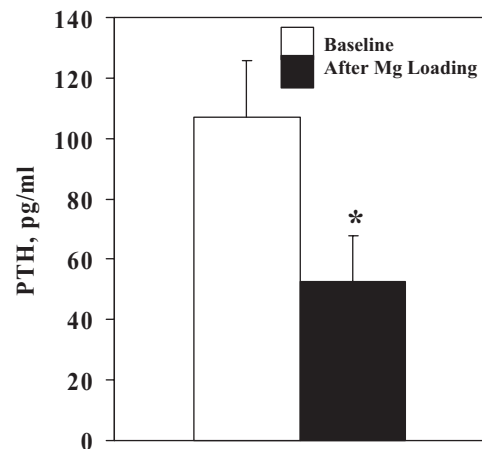


Fig. 9. Changes in serum PTH in WT mice supplemented with 100 mM Mg in the drinking water. Results are means  $\pm$  SE ( $n = 8$ ). \* $P < 0.05$  for comparison of the Mg-loaded value with the respective control value.



inhibitor of  $1\alpha$ -hydroxylase (31). In fact, this was the case, with all three genotypes exhibiting substantial increases in circulating  $1,25(\text{OH})_2\text{D}_3$  during Mg loading (Fig. 8). In the PTH KO and DKO mice, these increases ranged from three- to sixfold. Increases in circulating  $1,25(\text{OH})_2\text{D}_3$  of this magnitude could account, at least in part, for the changes in serum and urinary Ca in the three genotypes during Mg loading. In the case of the Ca-supplemented DKO mice, their impaired capacity to efficiently dispose of a Ca load via the kidney resulted in frank hypercalcemia in the setting of Mg loading.

Could the increases in serum  $1,25(\text{OH})_2\text{D}_3$  during Mg loading observed in our studies affect intestinal absorption or renal handling of Mg? There is both passive paracellular and active transcellular absorption and reabsorption of Mg in the intestine and kidney, respectively. Transcellular Mg transport is mediated, at least in part, by the recently discovered Mg-permeable channel TRPM6 in both the intestine and kidney (27, 29). Its expression does not appear to be increased by  $1,25(\text{OH})_2\text{D}_3$ . Therefore, transcellular transport of Mg would likely not be enhanced by the increase in  $1,25(\text{OH})_2\text{D}_3$  observed here. At the high concentration of Mg in the drinking water used in our studies to produce a Mg load, paracellular transport of Mg would be expected to dominate in the intestine and kidney. There is conflicting evidence regarding the effect of vitamin D on intestinal Mg absorption. Although pharmacological doses of vitamin D can enhance Mg absorption *in vivo* and *in vitro*, there do not appear to be substantial effects of more physiological changes in  $1,25(\text{OH})_2\text{D}_3$  (23), consistent with the failure of  $1,25(\text{OH})_2\text{D}_3$  to upregulate TRPM6 in the transcellular pathway. Vitamin D therapy in hypoparathyroid humans can enhance renal excretion of Mg without increasing intestinal absorption, thereby inducing negative Mg balance (17). This result contrasts with the increases in serum Mg observed during Mg loading in our studies [with the associated increases in serum  $1,25(\text{OH})_2\text{D}_3$ ] in the KO genotypes. Therefore, it is unlikely that the changes in serum and urinary Mg in the present studies were the result of vitamin D-mediated changes in intestinal absorption or renal reabsorption of Mg.

Could changes in bone turnover contribute to the changes in serum and urinary Mg as well as serum and urinary Ca observed here? As expected, the PTH-deficient PTH KO and DKO mice had modest reductions in serum alkaline phosphatase and osteocalcin, markers of bone turnover, and in urinary DPD, a marker of bone resorption. Mg loading had no effect on these parameters in the WT mice, whereas it increased osteocalcin by 15–20% in Ca-supplemented, Mg-loaded DKO mice and raised DPD by nearly 50% in both the Ca-supplemented PTH KO and DKO mice during Mg loading. The greater increase in bone resorption than in formation markers in the Mg-supplemented KO genotypes might result from the increase in  $1,25(\text{OH})_2\text{D}_3$  under these conditions and could have contributed to the hypercalcemia observed in these two genotypes in this experimental paradigm. Another contributory factor to the Mg-induced increase in bone resorption in the DKO mice could have been their markedly reduced CT level. Furthermore, it is possible that the enhanced bone resorption resulting from CT deficiency could have contributed to their marked hypercalcemia during Mg loading when they received calcium supplementation of their water, especially in view of their difficulty in renal clearance of calcium. In the wild-type mice, the decrease in PTH taking place during Mg loading may

have mitigated any effect of their increase in  $1,25(\text{OH})_2\text{D}_3$  on bone turnover.

In summary, loss of the full-length CaSR impairs the ability of DKO mice to mitigate the rise in serum Mg induced by Mg loading when the mice have been rendered normocalcemic by supplementation of their drinking water with Ca but not in nonsupplemented hypocalcemic mice. The loss of the CaSR in the TAL of the kidney may impair the ability of the DKO mice to inhibit renal tubular Mg reabsorption during Mg loading when supplemented with Ca. In general, however, loss of the CaSR in these mice appears to have less impact on Mg homeostasis than it did on Ca homeostasis in our previous studies. Notably, in this regard, the Ca-supplemented DKO mice develop marked hypercalcemia during Mg loading by a mechanism that is likely multifactorial. Contributory factors include 1) reduced ability to inhibit renal tubular reabsorption of Ca, 2) lack of Ca-stimulated CT secretion, 3) increased production of  $1,25(\text{OH})_2\text{D}_3$  in response to  $\text{P}_i$  depletion, which increases both gastrointestinal absorption and skeletal release of Ca owing to enhanced bone resorption, and 4) increased Ca bioavailability with  $\text{P}_i$  chelation by Mg. Whereas the wild-type mice can simultaneously coordinate and maintain calcium and magnesium homeostasis under these conditions, the DKO mice (and to a lesser extent the PTH KO mice) show substantially greater changes in serum Ca and Mg during Mg loading, indicating impaired homeostatic control of both divalent cations. Thus the CaSR and to some extent PTH facilitate the coordination of Ca and Mg homeostasis during stresses on these systems (in this case, Mg loading). Finally, the  $\text{P}_i$  depletion resulting from chelation of  $\text{P}_i$  in the gut by Mg is not widely recognized and should be borne in mind when patients receive Mg supplementation and would be expected to be particularly marked in people taking both Ca and Mg supplements.

#### GRANTS

These studies were supported by US Public Health Service Grant DK-078331 (to E. M. Brown), grants from the Drug Research Academy and Oticon Fonden (to A. R. B. Thomsen), and a grant from the Canadian Institutes for Health Research (D. Goltzman).

#### DISCLOSURES

None of the authors have any disclosures, financial or otherwise, that are relevant to this work.

#### AUTHOR CONTRIBUTIONS

S.J.Q., A.R.B.T., and E.M.B. contributed to the conception and design of the research; S.J.Q., A.R.B.T., O.I.E., and J.P. performed the experiments; S.J.Q., A.R.B.T., and K.B. analyzed the data; S.J.Q., A.R.B.T., O.I.E., J.P., K.B., D.G., and E.M.B. interpreted the results of the experiments; S.J.Q. and A.R.B.T. prepared the figures; S.J.Q., A.R.B.T., O.I.E., J.P., K.B., D.G., M.R.P., and E.M.B. edited and revised the manuscript; S.J.Q., A.R.B.T., O.I.E., D.G., M.R.P., and E.M.B. approved the final version of the manuscript; E.M.B. drafted the manuscript.

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