

The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion

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Kantham L, Quinn SJ, Egbuna OI, Baxi K, Butters R, Pang JL, Pollak MR, Goltzman D, Brown EM. The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. *Am J Physiol Endocrinol Metab* 297: E915–E923, 2009. First published July 28, 2009; doi:10.1152/ajpendo.00315.2009.—The calcium-sensing receptor (CaSR) controls parathyroid hormone (PTH) secretion, which, in turn, via direct and indirect actions on kidney, bone, and intestine, maintains a normal extracellular ionized calcium concentration (Ca^{2+}_o). There is less understanding of the CaSR's homeostatic importance outside of the parathyroid gland. We have employed single and double knockout mouse models, namely mice lacking PTH alone (CaSR^{+/+} PTH^{-/-}, referred to as C⁺P⁻), lacking both CaSR and PTH (CaSR^{-/-} PTH^{-/-}, C⁻P⁻) or wild-type (CaSR^{+/+} PTH^{+/+}, C⁺P⁺) mice to study CaSR-specific functions without confounding CaSR-mediated changes in PTH. The mice received three hypercalcemic challenges: an oral Ca^{2+} load, injection or constant infusion of PTH via osmotic pump, or a phosphate-deficient diet. C⁻P⁻ mice show increased susceptibility to developing hypercalcemia with all three challenges compared with the other two genotypes, whereas C⁺P⁻ mice defend against hypercalcemia similarly to C⁺P⁺ mice. Reduced renal Ca^{2+} clearance contributes to the intolerance of the C⁻P⁻ mice to Ca^{2+} loads, as they excrete less Ca^{2+} at any given Ca^{2+}_o than the other two genotypes, confirming the CaSR's direct role in regulating renal Ca^{2+} handling. In addition, C⁺P⁺ and C⁺P⁻, but not C⁻P⁻, mice showed increases in serum calcitonin (CT) levels during hypercalcemia. The level of 1,25(OH)₂D₃ in C⁻P⁻ mice, in contrast, was similar to those in C⁺P⁻ and C⁺P⁺ mice during an oral Ca^{2+} load, indicating that increased 1,25(OH)₂D₃ production cannot account for the oral Ca^{2+} -induced hypercalcemia in the C⁻P⁻ mice. Thus, CaSR-stimulated PTH release serves as a “floor” to defend against hypocalcemia. In contrast, high- Ca^{2+}_o -induced inhibition of PTH is not required for a robust defense against hypercalcemia, at least in mice, whereas high- Ca^{2+}_o -stimulated, CaSR-mediated CT secretion and renal Ca^{2+} excretion, and perhaps other factors, serve as a “ceiling” to limit hypercalcemia resulting from various types of hypercalcemic challenges.

serum calcium; calcitonin; kidney; vitamin D; urinary calcium; diuretic

THE EXTRACELLULAR CALCIUM (Ca^{2+}_o)-SENSING RECEPTOR (CaSR) plays key roles in Ca^{2+}_o homeostasis (5). A large body of evidence indicates that the CaSR in the parathyroid chief cell, where it is expressed at arguably the highest level in the body, plays crucial roles in Ca^{2+} homeostasis by inhibiting parathy-

roid hormone (PTH) secretion (6, 22, 31), PTH gene expression (26), and parathyroid cellular proliferation (22, 45), as well as by upregulating the vitamin D receptor (41). Key pieces of evidence in this regard are the excessive, unregulated PTH secretion and parathyroid cell growth in mice homozygous for knockout (KO) of the CaSR gene (22) as well as in humans homozygous for inactivating mutations of the CaSR as part of the syndrome of neonatal severe hyperparathyroidism (NSHPT) (5, 21, 27). Furthermore, allosteric activators (“calcimimetics”) (31) and inhibitors (“calcilytics”) of the CaSR (18), as expected, inhibit and stimulate parathyroid function, respectively.

In addition to being present in the parathyroid, the CaSR is also expressed in other tissues participating in Ca^{2+}_o homeostasis, including the calcitonin (CT)-secreting C-cells of the thyroid gland (15, 17) and several sites along the renal tubule (38, 39). Available data support the CaSR's role in mediating high- Ca^{2+}_o -stimulated CT secretion (15, 17, 28), in promoting renal Ca^{2+} excretion in response to hypercalcemia (1, 2, 11, 13, 14), and in regulating the secretion of parathyroid hormone-related protein (PTHrP) and the transport of calcium in breast epithelial cells (44). Although the CaSR is present in bone cells (8, 23, 29, 47), as well as in epithelial cells of the small and large intestines (10, 16), the predominant cells that express it and its roles in regulating bone turnover and intestinal function, including Ca^{2+} absorption, are not fully understood. Recent studies, however, using conditional knockout (KO) mouse models have implicated the CaSR as playing key roles in chondrocytes and osteoblasts (9).

The neonatal lethality of the homozygous CaSR KO mice has limited their utility for detailed studies of the CaSR's physiological roles in vivo (22). However, several other mouse models are available for such investigations. Two such models “rescue” the homozygous CaSR KO mice by crossing heterozygous CaSR KO mice with mice in which either the parathyroid gland (42) or the PTH gene (24) has been knocked out. Studies using these two models have established that the CaSR contributes importantly to the fine control of the serum Ca^{2+} concentration but have not evaluated in any detail the importance of the CaSR in C-cells and kidney and other tissues in maintaining Ca^{2+}_o homeostasis not only in the absence, but also in the presence of PTH (e.g., given via infusion). As noted above, mice with conditional KO in various tissues offer an additional approach to studying the CaSR's roles in vivo (9). However, the conditional KO mice lack the advantages that global KO mice offer, i.e., the ability to observe homeostatic responses in the absence of the compensatory role(s) played by

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CaSR present in nontargeted tissues (see DISCUSSION for more details).

In the present studies we have utilized as an experimental model the exon 5 CaSR KO mice rescued by deletion of the PTH gene (CaSR^{-/-} PTH^{-/-}, referred to as C⁻P⁻ mice), utilizing mice with KO of PTH alone (CaSR^{+/+} PTH^{-/-}, C⁺P⁻) or wild-type mice (CaSR^{+/+} PTH^{+/+}, C⁺P⁺) as controls to dissect the relative roles of the CaSR in parathyroid, kidney and C-cell in maintaining Ca²⁺_o homeostasis. The results indicate that, while CaSR-regulated PTH secretion provides a “floor” that defends against hypocalcemia, it is not needed to mount a robust and effective defense against hypercalcemic challenges, such as an oral calcium load or PTH infusion. The near total lack of Ca²⁺_o-stimulated CT secretion in the C⁻P⁻ mice as well as their impaired ability to upregulate renal Ca²⁺ excretion, especially in the presence of PTH, are likely important contributors to their increased susceptibility to hypercalcemia induced by increased Ca²⁺ ingestion, by PTH injection or PTH infusion by minipump or by phosphate depletion. These results illustrate the CaSR's second key homeostatic role, as a “ceiling” that defends effectively against hypercalcemia even in the absence of CaSR-mediated suppression of PTH.

MATERIALS AND METHODS

Generation of C⁺P⁺, C⁺P⁻, and C⁻P⁻ mouse lines. The two parental strains of CaSR^{-/-} mice and PTH^{-/-} mice were generated by homologous recombination in embryonic stem cells (22, 30). 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 C⁺P⁺, C⁺P⁻ and C⁻P⁻ mice. We maintained lines of C⁻P⁻, C⁺P⁻, and C⁺P⁺ mice that had been extensively back-crossed 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 periodically tested to ensure stability of the biochemical phenotypes.

KO mice. The WT (C⁺P⁺), PTH KO (C⁺P⁻), and PTH and CaSR double-KO (C⁻P⁻) mice were maintained in microisolator cages in a Brigham and Women's Hospital animal facility. We routinely genotyped and screened the mice biochemically to ensure the authenticity and stability of their genetic backgrounds. The PCR primers and genotyping protocols were applied as described previously (22, 30).

In vivo experiments. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School and were in accordance with the NIH *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 (ARCM). As described below (see RESULTS), in some experiments, C⁺P⁻ and C⁻P⁻ mice were rendered hypocalcemic (owing to their hypoparathyroidism) by feeding them standard mouse chow (with 0.8% Ca²⁺ wt/wt and 0.4% phosphate; Harlan-Teklad TD99224; Harlan-Teklad, Madison, WI) as well as drinking water with no added calcium (referred to as 0% Ca²⁺ water). In other experiments, the three genotypes were rendered equivalently normocalcemic by feeding the C⁻P⁻ and C⁺P⁻ mice the same chow along with drinking water supplemented with CaCl₂ [0.75% (wt/vol) and 1.5% (wt/vol), respectively]. Note that the percentage of calcium expressed as wt/vol refers to the weight of the CaCl₂ salt added to the water and not to elemental Ca²⁺. 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 urine Ca²⁺ (Eagle Diagnostics, De Soto, TX), urine creatinine, Creatinine Analyzer 2 (Beckman Coulter, Galway, Ireland), serum creatinine, alkaline phosphatase (ALP) (Stanbio Laboratory, Boerne, TX), 1,25(OH)₂D₃, RIA (Immunodiagnosics Systems, Fountain Hills, AZ), urine deoxypyridinoline (DPD) crosslinks (Quidel, San Diego, CA), osteocalcin and CT (Alpco Diagnostics, Salem, NH), and genotyping, DNeasy kit and primers (Qiagen).

Osmotic minipumps. Miniosmotic pumps (model 1002) and human PTH₁₋₃₄ were purchased from Alzet (Cupertino, CA) and Bachem (Torrance, CA), respectively. Pumps were filled with hPTH₁₋₃₄ dissolved in PBS with 2% cysteine (adjusted to pH 4.3 with addition of 20 mM acetic acid) and preconditioned by incubation in the same buffer overnight at 37°C. Mice were anesthetized briefly with isoflurane, during which a subcutaneous pocket was prepared on the lower dorsal surface for subsequent implantation of the minipump. After implantation, the incision was clipped, and postoperative analgesia was administered as needed for the first 24 h. The mice were kept in separate cages to avoid one mouse damaging the minipump implanted on another one by chewing. The pump volume, pumping rate, and duration were ~100 μl, 0.25 μl/h, and 14 days, respectively.

Statistics. Statistical analyses were carried out by unpaired *t*-testing 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 <0.05 was taken to indicate statistical significance. In general, each experiment was performed at least twice, and results were pooled if the experimental design was identical.

RESULTS

Response of mice to oral Ca²⁺ loading. We initially assessed the capacity of the C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice to maintain normocalcemia in response to oral Ca²⁺ loading achieved by increasing the concentration of Ca²⁺ in the water. Figure 1 shows that, in contrast to the normocalcemic C⁺P⁺ mice, the C⁺P⁻ and C⁻P⁻ mice were both hypocalcemic to a similar degree (6–7 mg/dl) when maintained on chow with 0.8% Ca²⁺ and 0% Ca²⁺ water. Therefore, as noted previously (22, 24, 42), loss of the CaSR did not protect against the development of hypocalcemia in the absence of PTH. When the mice were provided with drinking water containing 1% (wt/vol) CaCl₂ (referred to hereafter as 1% Ca²⁺ water), the C⁺P⁻ mice exhibited an increase in serum Ca²⁺ to 8.3 mg/dl, whereas the C⁻P⁻ mice showed a substantially greater increase in serum Ca²⁺ under these conditions, to 10–11 mg/dl (*P* < 0.05). When the Ca²⁺ content of the water was increased to 2% (referred to as 2% Ca²⁺ water), the C⁻P⁻ mice became overtly hypercalcemic (~14 mg/dl), but the C⁺P⁻ mice were able to defend effectively against hypercalcemia, maintaining a serum Ca²⁺ of 9.6 mg/dl. The C⁺P⁺ mice remained normocalcemic throughout the study. The mice drank equivalent amounts of water (3.2–3.6 ml/25 g mouse per day), and serum creatinine concentrations after 1 wk on the 2% Ca²⁺ water did not differ among the three genotypes and were unchanged relative to the corresponding levels when the mice were receiving 0% Ca²⁺ water.

Possible hormonal factors contributing to oral Ca²⁺-induced hypercalcemia in the C⁻P⁻ mice. To explore possible hormonal and other factors that could contribute to the exaggerated hypercalcemic response of the C⁻P⁻ mice to receiving

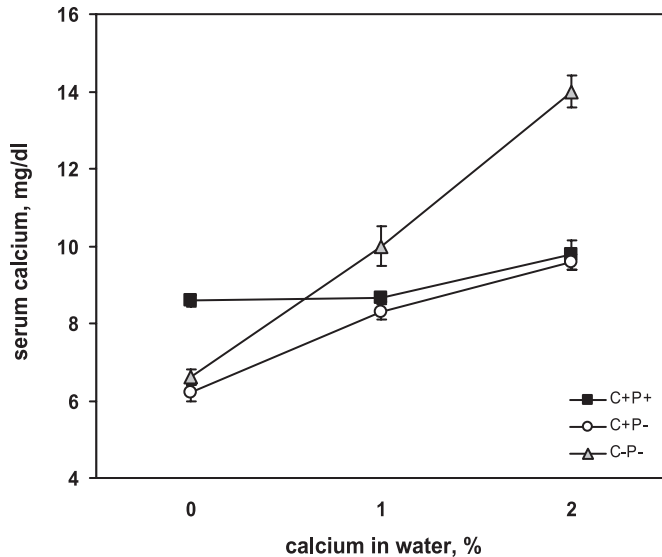


Fig. 1. Serum Ca²⁺ concentration in wild-type (C⁺P⁺), parathyroid hormone (PTH) knockout (C⁺P⁻), and PTH and calcium-sensing receptor (CaSR) double-knockout (C⁻P⁻) mice as a function of Ca²⁺ in the drinking water. All mice received chow with 0.8% Ca²⁺ throughout the study. After 1 wk on 0% Ca²⁺ water, blood and urine samples were obtained, and 1% Ca²⁺ (as CaCl₂) was added to the water. After 1 wk on 1% Ca²⁺ water, blood and urine samples were obtained, and Ca²⁺ in the water was changed to 2%. Blood and urine samples were obtained a third time 1 wk later. The serum Ca²⁺ concentration of the C⁻P⁻ mice was significantly higher than in the C⁺P⁻ mice with both 1% and 2% Ca²⁺ water and significantly higher than that of the C⁺P⁺ mice with 2% Ca²⁺ water (means ± SE, *P* < 0.01, *n* = 12–16 in each genotype).

2% Ca²⁺ water, we measured serum 1,25(OH)₂D₃ levels in the three genotypes of mice receiving 0 or 2% Ca²⁺ water (Fig. 2). The C⁺P⁻ and C⁻P⁻ mice had slightly but not significantly lower levels of 1,25(OH)₂D₃ than the C⁺P⁺ mice at baseline, and the C⁺P⁻ and C⁻P⁻ mice did not differ in their 1,25(OH)₂D₃ levels. When mice were receiving 2% Ca²⁺ water, the 1,25(OH)₂D₃ levels of all three genotypes remained nearly constant, indicating that a higher level of 1,25(OH)₂D₃ was not a contributor to the difference in the serum Ca²⁺

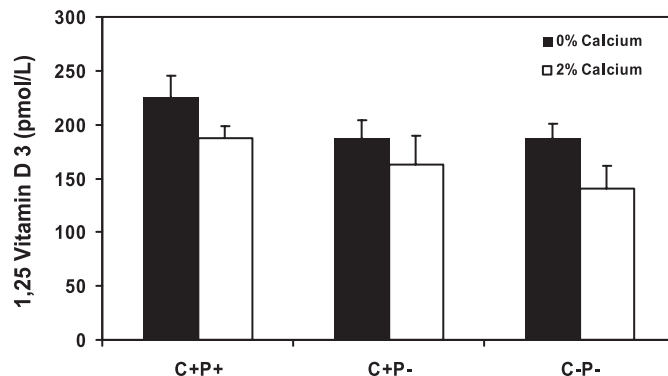


Fig. 2. Serum 1,25(OH)₂D₃ levels in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice receiving 0 or 2% Ca²⁺ water. Mice were fed normal chow and 0% Ca²⁺ water for 1 wk, and blood was drawn for measurement of serum levels of 1,25(OH)₂D₃ as described in MATERIALS AND METHODS. Mice then received normal chow and 2% Ca²⁺ water for 1 wk, and measurements of 1,25(OH)₂D₃ were repeated. The level of 1,25(OH)₂D₃ in the C⁺P⁺ mice on 0% Ca²⁺ water was significantly higher than that in either C⁺P⁻ or C⁻P⁻ mice. Values of 1,25(OH)₂D₃ did not differ significantly among the 3 genotypes when receiving 2% Ca²⁺ in the drinking water. (means ± SE, *n* = 8–16 in each genotype).

between C⁺P⁻ and C⁻P⁻ mice receiving 2% Ca²⁺ water. The high bioavailability of the Ca²⁺ from the CaCl₂ in the drinking water, with resultant impairment of gastrointestinal phosphate absorption and hypophosphatemia, may have contributed to the lack of suppression of the 1,25(OH)₂D₃ in the C⁺P⁺ mice receiving 2% Ca²⁺ water. Indeed, serum phosphate in the C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice decreased from 7.5 ± 0.36 to 4.1 ± 0.3, from 9.2 ± 0.47 to 4.9 ± 0.43, and from 9.8 ± 0.73 to 3.9 ± 0.27 mg/dl, respectively, when the mice changed from 0 to 2% Ca²⁺ water.

Figure 3 shows the relationship between serum Ca²⁺ and CT concentrations in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice administered 0, 1, or 2% Ca²⁺ water. The trend lines fitting the data for the C⁺P⁺ and C⁺P⁻ mice on the one hand, and the C⁻P⁻ mice on the other, were provided as visual aids to highlight the differences in the responses between the genotypes. Statistical analyses were carried out over specific ranges of serum calcium concentration as described below. In contrast to the C⁺P⁺ and C⁺P⁻ mice, which showed a steep increase in serum CT between ~9 and 12 mg/dl, the C⁻P⁻ mice exhibited minimal if any changes in serum CT. The CT values for the three genotypes over a near-physiological range of serum calcium concentrations (8–10 mg/dl) as well as for >10 mg/dl were compared, and the CT values of C⁻P⁻ mice were significantly lower (*P* < 0.01) than those of C⁺P⁻ and C⁺P⁺ mice.

To assess responsiveness of the mice to CT, they were maintained on 2% Ca²⁺ water for 1 wk and then injected with a single dose of 150 ng/g body wt salmon CT (Fig. 4). The CT injection produced a substantial fall in the serum Ca²⁺ concentration of the C⁻P⁻ mice. Regardless of whether it was expressed as absolute decrement (4.7 mg/dl for C⁻P⁻ vs. 2.1 mg/dl for C⁺P⁻ and 1.7 mg/dl for C⁺P⁺) or as percentage decrease (29.8% for C⁻P⁻ vs. 21.3% for C⁺P⁻ and 15.0% for C⁺P⁺), the drop in serum calcium concentration for the C⁻P⁻ mice was as great or greater than that in the C⁺P⁺ and C⁺P⁻ mice. This result documented that impaired responsiveness to endogenous CT could not account for the hypercalcemia in the C⁻P⁻ mice administered 2% Ca²⁺ water. CT produced only a

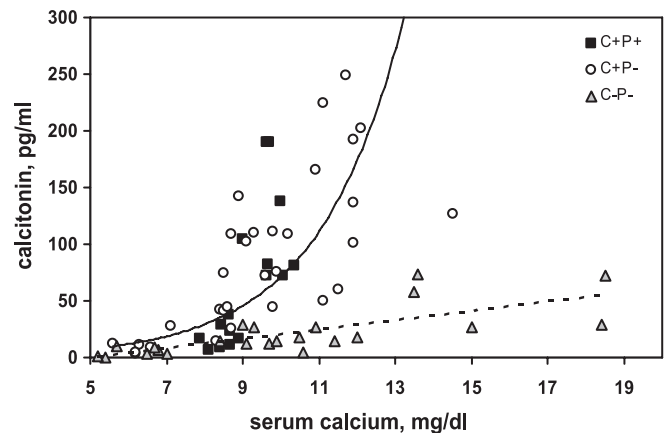


Fig. 3. Serum calcitonin (CT) levels in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice as a function in serum Ca²⁺ concentration. Mice were maintained on standard chow and 0% Ca²⁺ water for 1 wk, 1% Ca²⁺ water for 1 wk and finally, 2% Ca²⁺ water for a 3rd wk. Serum samples were obtained at the end of each of the 3 wk, and levels of Ca²⁺ and CT were determined as described in MATERIALS AND METHODS. Data are plotted as serum Ca²⁺ concentration in any given serum sample vs. the CT concentration in that sample. Trend lines represent C⁺P⁻ and C⁺P⁺ (solid) and C⁻P⁻ (dotted).

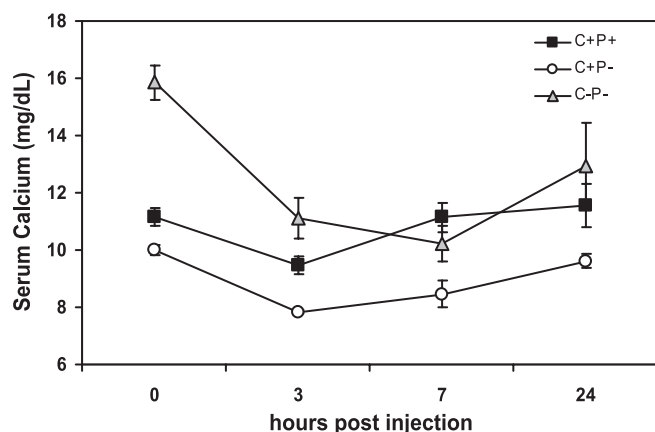


Fig. 4. Serum Ca^{2+} concentrations in C^+P^+ , C^+P^- , and C^-P^- mice receiving 2% Ca^{2+} water as a function of time after receiving an injection of CT. Mice were maintained on normal chow and 2% Ca^{2+} water for 1 wk. They then received an SC injection of salmon CT (150 ng/g body wt), and serum samples were obtained for determination of serum Ca^{2+} concentration as before at times shown. The maximal decrement in the absolute level of serum Ca^{2+} concentration after the CT injection in C^-P^- mice was greater than in C^+P^+ or C^+P^- mice ($P < 0.05$).

small decrement in serum Ca^{2+} in the C^+P^+ mice, presumably because they were able to mount a sufficient PTH response to prevent hypocalcemia.

Renal contribution to oral Ca^{2+} -induced hypercalcemia in the C^-P^- mice. Available data in humans suggest that a key homeostatic action of the CaSR is to regulate renal tubular reabsorption of calcium, most likely through actions in the thick ascending limb (TAL) (1, 2, 12, 14). We studied, therefore, the impact of loss of the CaSR on renal calcium handling in the C^-P^- mice. Figure 5 shows the relationship between serum and urine Ca^{2+} concentrations in the three genotypes of mice when they were receiving 0, 1, or 2% Ca^{2+} water. The urinary Ca^{2+} data are expressed as milligrams of Ca^{2+} per milligram of creatinine, because it was difficult to obtain full 24-h collections. There was a steep rise in the urinary Ca^{2+} -to-creatinine ratio when serum Ca^{2+} increased to ~ 10 mg/dl in both the C^+P^+ and C^+P^- mice. The similar responses of these two genotypes illustrates that suppression of PTH was not required to observe the increase in urinary Ca^{2+} excretion accompanying elevations in serum Ca^{2+} , even within the normal range. For the C^-P^- mice, many of the mice required Ca^{2+} concentrations of 12–15 mg/dl to achieve levels of urinary Ca^{2+} excretion equivalent to those of the C^+P^- and C^+P^+ mice when their serum Ca^{2+} levels rose to ~ 10 mg/dl. At serum Ca^{2+} concentrations between 8 and 10 mg/dl, corresponding to a physiological range of serum Ca^{2+} concentrations, the urinary Ca^{2+} concentrations of the C^+P^+ , C^+P^- , and C^-P^- mice were 1.2 ± 0.18 , 3.6 ± 0.27 , and 1.6 ± 0.25 mg/mg creatinine, respectively ($P < 0.01$ for C^-P^- vs. C^+P^-). The urinary Ca^{2+} concentrations of the C^+P^- and C^-P^- mice also differed significantly even when they were hypocalcemic on 0% Ca^{2+} water (0.33 ± 0.038 and 0.17 ± 0.03 , respectively, $P < 0.001$).

Responses of bone turnover markers to oral Ca^{2+} loading. Figure 6 shows that the levels of urinary deoxy pyridinoline (DPD) in the C^+P^- and C^-P^- mice were lower than those of the C^+P^+ mice when receiving 0% Ca^{2+} water and that the two KO genotypes had nearly equivalent levels of DPD. When

they received 2% Ca^{2+} water, the levels of DPD in the two KO genotypes did not change, suggesting that a difference in bone resorption between these two genotypes could not account for the greater hypercalcemic response of the C^-P^- mice to an oral Ca^{2+} load. Serum osteocalcin (OC) concentrations in the three genotypes were similar when receiving 0% Ca^{2+} water. When they received 2% Ca^{2+} water, however, there were significant reductions in OC in the C^+P^+ and C^+P^- mice but not in the C^-P^- mice, suggesting a role for the CaSR in regulating osteoblast function as assessed by circulating levels of OC. In addition, the results suggest that impaired bone formation did not contribute to the hypercalcemia of the C^-P^- mice receiving 2% Ca^{2+} water.

Effect of PTH administration on C^+P^+ , C^+P^- , and C^-P^- mice. Figure 7 shows that injecting a single dose of PTH (500 ng/g body wt) had essentially no effect on the C^+P^+ mice but increased serum Ca^{2+} substantially in the C^+P^- and C^-P^- mice when receiving 0% Ca^{2+} water. The increase in serum Ca^{2+} in the C^-P^- mice, however, was twice that in the C^+P^- mice and produced frank hypercalcemia ($P < 0.01$), again showing the enhanced sensitivity of the C^-P^- mice to agents inducing hypercalcemia. The action of the PTH was, as expected, of short duration. Therefore, we investigated the effect of infusion with PTH_{1-34} by osmotic minipump on the two KO genotypes by collecting samples at days 3 and 6 after the initiation of the infusion. We did not include the C^+P^+ mice in these studies because of the confounding effect of changes in their endogenous PTH secretion that might result from infusion

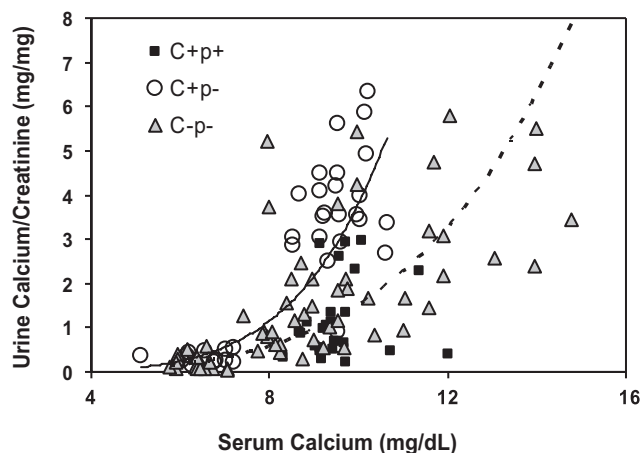


Fig. 5. Urine Ca^{2+} -to-creatinine ratio as a function of serum Ca^{2+} concentration in C^+P^+ , C^+P^- , and C^-P^- mice maintained on 0, 1, and then 2% Ca^{2+} water. Mice were initially maintained on normal chow and 0% Ca^{2+} water for 1 wk. Blood and spot urine specimens were collected on the 7th day. The drinking water was then changed to 1% Ca^{2+} for 7 days, and blood and urine specimens again obtained on day 7. Finally, the water was changed to 2% Ca^{2+} for 7 days, and urine and blood collections were again obtained on day 7. Serum Ca^{2+} and urine Ca^{2+} and creatinine were measured as in MATERIALS AND METHODS, and results are plotted as serum Ca^{2+} concentration vs. corresponding Ca^{2+} -to-creatinine ratio. Urine Ca^{2+} levels in C^-P^- mice receiving 0% Ca^{2+} water were significantly lower than those in C^+P^+ mice under the same conditions (0.33 ± 0.038 and 0.17 ± 0.03 , respectively, $P < 0.001$). The urine Ca^{2+} concentration in C^+P^- and C^-P^- mice with serum Ca^{2+} concentrations between 8 and 10 mg/dl also differed significantly (3.6 ± 0.27 vs. 1.6 ± 0.25 mg/dl, respectively, $P < 0.01$). It was not possible to compare urinary Ca^{2+} concentration in C^+P^+ and C^+P^- genotypes at higher Ca^{2+} concentrations because of the difficulty in raising serum Ca^{2+} concentration above 10 mg/dl in C^+P^- (and C^+P^+) mice (means \pm SE, $n = 8$ –12 in each genotype). Trend lines represent C^+P^- (solid) and C^-P^- (dotted).

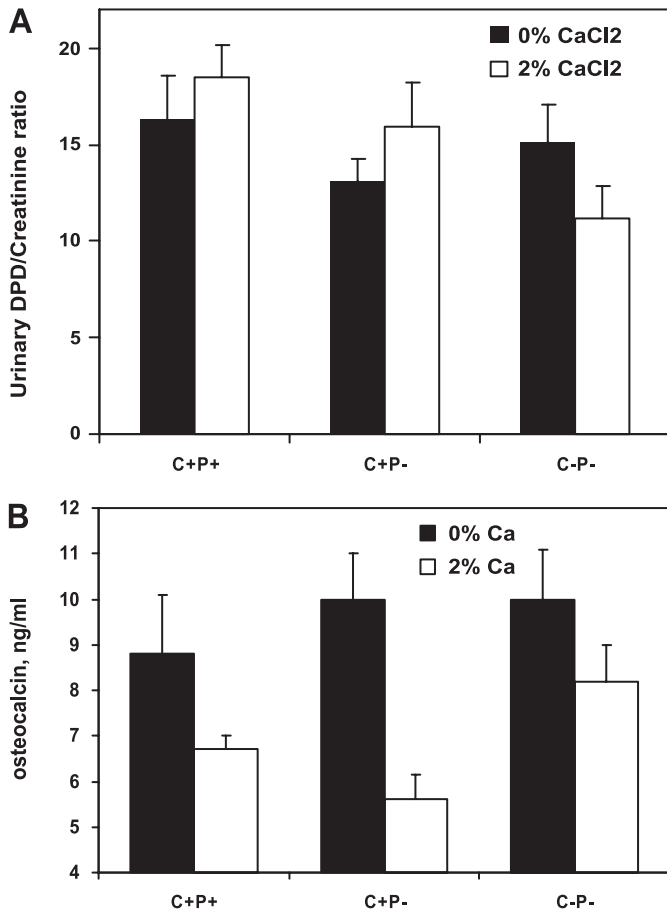


Fig. 6. Urinary deoxypyridinolines (DPD; A) and serum osteocalcin (OC) concentrations (B) in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice receiving 0 or 2% Ca²⁺ water. Mice were maintained for 1 wk on each concentration of Ca²⁺ in the water, and serum and urine specimens were obtained on the last day of each of the 2 wk. Urinary DPD and creatinine as well as serum OC were measured as in MATERIALS AND METHODS. There were no significant differences in DPD levels of C⁻P⁻ and C⁺P⁻ mice on 0 or 2% Ca²⁺ water, and changes in DPD within genotypes also did not change significantly when calcium in the drinking water was increased from 0 to 2%. Serum OC levels were not significantly different among the 3 genotypes when receiving 0% Ca²⁺ water. OC levels for C⁺P⁺ and C⁺P⁻ mice dropped significantly ($P < 0.05$) when mice received 2% Ca²⁺ water; however, OC levels for C⁻P⁻ mice showed no significant decline when mice received 2% Ca²⁺ water ($P > 0.5$) (means \pm SE, $n = 8-12$ in each genotype for both DPD and OC).

of PTH₁₋₃₄ and resultant increases in serum Ca²⁺. Figure 8 shows that the C⁻P⁻ mice had substantially greater hypercalcemic responses to infused PTH at any given dose, reaching levels of serum Ca²⁺ as high as 20 mg/dl at the highest dose tested (0.08 mg·kg⁻¹·day⁻¹). The levels of serum Ca²⁺ were significantly higher in the C⁻P⁻ than in the C⁺P⁻ mice receiving 0.04 mg·kg⁻¹·day⁻¹ at 3 and 6 days after initiating the infusion ($P < 0.05$), documenting the increased propensity of the C⁻P⁻ mice to PTH-induced hypercalcemia.

Figure 9 shows the relationship between serum and urine Ca²⁺ during the PTH₁₋₃₄ infusion in the C⁻P⁻ and C⁺P⁻ mice when the results were expressed as urinary Ca²⁺-to-creatinine ratios as a function of serum Ca²⁺ obtained on the same day as the urine for both the 3- and 6-day time points. There was a marked flattening of the slope of the relationship between serum and urine Ca²⁺ in C⁻P⁻ compared with C⁺P⁻ mice,

reflecting the very modest increase in urine Ca²⁺ in the C⁻P⁻ mice with escalating doses of PTH₁₋₃₄, despite the accompanying increases in serum Ca²⁺ to as high as 20 mg/dl. Five mice per genotype for the doses of 0.02 and 0.04 mg·kg⁻¹·day⁻¹ and two mice per genotype for the dose of 0.08 mg·kg⁻¹·day⁻¹ were implanted with pumps. Note that there are four points each for urinary calcium concentration for the C⁺P⁻ and C⁻P⁻ mice receiving 0.08 mg·kg⁻¹·day⁻¹ PTH₁₋₃₄ because each mouse was studied on 2 days (days 3 and 6 of the infusion). Despite their higher level of serum Ca²⁺ when receiving 0.04 mg·kg⁻¹·day⁻¹ of PTH, the urinary Ca²⁺ concentration of the C⁻P⁻ mice was lower than that in the C⁺P⁻ mice. Moreover, the urinary Ca²⁺ concentrations for the PTH-infused mice at serum calcium concentrations corresponding to 8–10 mg/dl were 0.903 ± 0.250 and 0.382 ± 0.144 for C⁺P⁻ and C⁻P⁻, respectively. The difference in the response between the two KO genotypes is highly significant ($P < 0.006$).

Effect of phosphate depletion on serum Ca²⁺ concentration in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice. Figure 10 shows the effect of administering a phosphate-deficient diet (nominally zero phosphate in the food and water) on serum Ca²⁺ and phosphate in mice of the three genotypes. There was a markedly greater increase in serum Ca²⁺ (and decrease in serum phosphate) in the C⁻P⁻ compared with the C⁺P⁻ mice (increments in serum Ca²⁺ of ~3 and 10 mg/dl, respectively, above the level observed with 0% Ca²⁺ water; $P < 0.01$), whereas the C⁺P⁺ mice only showed modest increases in serum Ca²⁺ (~1–2 mg/dl above the baseline shown in Fig. 1). Serum 1,25(OH)₂D₃ levels rose to comparable extents in all three genotypes ($1,193 \pm 124$, $1,076 \pm 116$, and $1,152 \pm 201$ pmol/l, respectively, in the C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice).

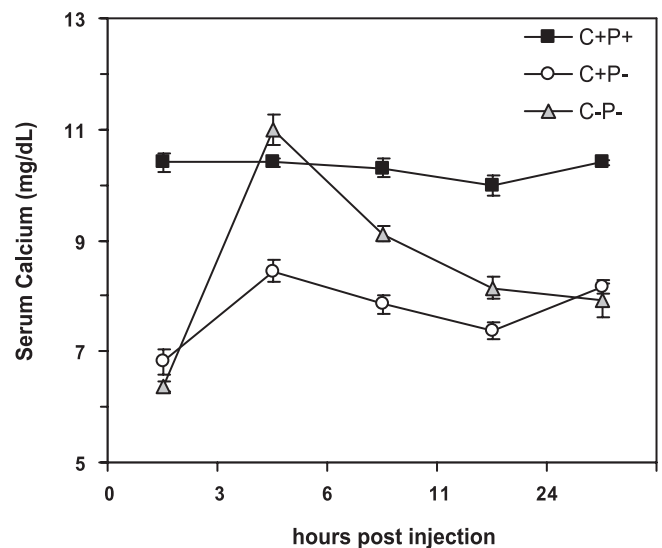


Fig. 7. Effect of injection with 500 ng/g body wt of hPTH₁₋₃₄ on serum Ca²⁺ of C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice. Mice received 0% Ca²⁺ water for 7 days prior to the experiment. Serum was obtained just before PTH injection and then 3, 6, 11, and 24 h later for measurement of serum Ca²⁺ concentration. Serum Ca²⁺ was unchanged in C⁺P⁺ mice. It increased significantly in both C⁺P⁻ and C⁻P⁻ mice, but the increment was greater in C⁻P⁻ mice at 3 ($P < 0.1$), 6 ($P < 0.1$), and 11 h ($P < 0.5$) after injection (means \pm SE, $n = 4$ for each genotype).

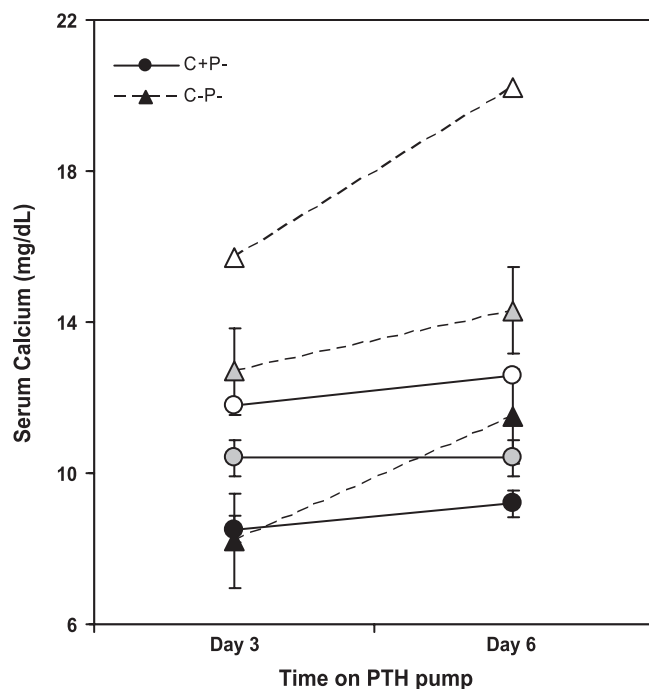


Fig. 8. Effect of infusion with hPTH₁₋₃₄ via minipump on serum Ca²⁺ in C⁺P⁻ (circles) and C⁻P⁻ (triangles) mice. Mice were maintained on normal chow and 0% Ca²⁺ water for 1 wk. Osmotic minipumps were then implanted sc, and hPTH₁₋₃₄ was infused at 0.02, 0.04, or 0.08 mg·kg⁻¹·day⁻¹ as denoted by solid, shaded, and open symbols, respectively. Serum Ca²⁺ concentrations were determined on *days 3* and *6* postimplantation ($n = 5$ for mice receiving 0.02 and 0.04 mg·kg⁻¹·day⁻¹ and $n = 2$ for mice receiving 0.08 mg·kg⁻¹·day⁻¹). Mean serum Ca²⁺ for noninfused mice ($n = 13-16$) after 1 wk on normal chow and 0% Ca²⁺ water, as determined on an independent set of mice, were 6.8 for C⁺P⁻ mice and 6.3 for C⁻P⁻ mice. Serum Ca²⁺ was significantly higher in C⁻P⁻ than in C⁺P⁻ mice infused with 0.04 mg·kg⁻¹·day⁻¹ for either 3 ($P < 0.05$) or 6 days ($P < 0.02$). Although serum Ca²⁺ was markedly higher in C⁻P⁻ mice than in C⁺P⁻ mice infused with 0.08 mg·kg⁻¹·day⁻¹, the small number of animals studied precluded statistical analysis.

DISCUSSION

The goal of the present study was to examine the role of the CaSR in maintaining Ca²⁺ homeostasis in the absence of PTH and, consequently, CaSR-regulated PTH secretion. Several points are germane in this regard. We utilized mice with KO of exon 5 of the CaSR, which, in the homozygous state when their parathyroid glands are intact, die of severe hypercalcemia and hyperparathyroidism within the first few weeks of life (22). This result supports the conclusion that this model represents a complete or nearly complete KO of the CaSR in the parathyroid and, perhaps, other tissues involved in Ca²⁺ homeostasis. However, subsequently it has become clear that a splice variant of the CaSR arising from splicing out of exon 5 of the *Casr* gene can be formed in some tissues under normal circumstances as well as in mice homozygous for KO of exon 5 of the *Casr* (33, 34). It has not been possible to express high levels of this truncated receptor on the cell surface of heterologous cell systems, preventing direct documentation that it actually has biological activity. At the same time, it appears that the chondrocytes from exon 5 CaSR KO mice still respond to Ca²⁺, providing indirect evidence that the CaSR lacking exon 5 may have biological activity in some tissues (40). Recent studies strongly suggest that the CaSR lacking exon 5 in the

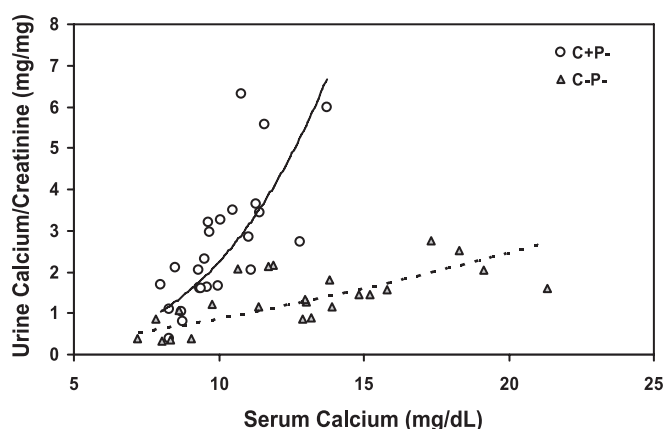


Fig. 9. Effect of PTH infusion on urinary Ca²⁺ concentration as a function of serum Ca²⁺ in C⁻P⁻ and C⁺P⁻ mice. Two independent urine samples were obtained on the same days as the serum samples in the experiment shown in Fig. 8, and urine Ca²⁺-to-creatinine ratios are plotted as a function of serum Ca²⁺ concentration. Urine Ca²⁺ values were significantly lower in C⁻P⁻ mice than in C⁺P⁻ mice receiving 0.04 mg·kg⁻¹·day⁻¹ PTH, despite significantly higher serum Ca²⁺ concentrations of C⁻P⁻ mice, demonstrating their relative hypocalciuria. C⁻P⁻ mice exhibited only a modest increase in urinary Ca²⁺ concentration with increasing rates of PTH infusion, in contrast to the marked increase in urinary Ca²⁺ concentration in C⁺P⁻ mice, as serum Ca²⁺ concentration rose in response to PTH infusion. There were significant differences in urinary Ca²⁺ concentration ($P < 0.05$) between C⁺P⁻ and C⁻P⁻ mice at the 0.02, 0.04, and 0.08 mg·kg⁻¹·day⁻¹ infusion rates when data from *days 3* and *6* were pooled. Trend lines represent C⁺P⁻ (solid) and C⁻P⁻ (dotted).

global exon 5 CaSR KO rescues the otherwise severe cartilage and bone phenotypes that results from tissue-selective KO of the CaSR in chondrocytes and osteoblasts (7, 9). However, as shown in our results, and discussed further below, mice with global KO of exon 5 of the *Casr* gene have marked abnormalities in the control of CT and renal Ca²⁺ handling in response to hypercalcemia (22), suggesting that the full-length CaSR, which is lost in the exon 5 KO mice, is a key mediator of these

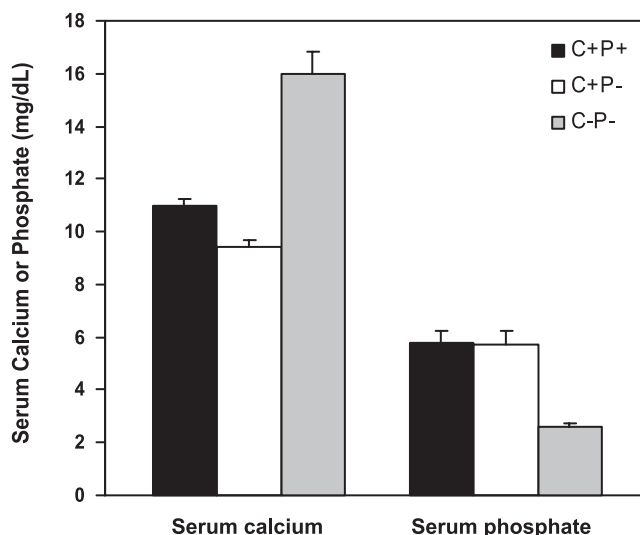


Fig. 10. Effect of phosphate-deficient diet on serum Ca²⁺ and phosphate concentrations in C⁺P⁺, C⁺P⁻, and C⁻P⁻ mice. Mice were maintained on a nominally phosphate-free diet with 0% Ca²⁺ water for 6 days. Serum was then obtained for measurements of Ca²⁺ and phosphate as described in MATERIALS AND METHODS. Serum Ca²⁺ levels differed significantly among all genotypes ($P < 0.01$) (means SEM, $n = 8$ in each genotype).

two biological actions of high Ca^{2+} as well as of Ca^{2+} -regulated PTH secretion and parathyroid cellular proliferation.

Our results are relevant to the CaSR's roles in defending against not only hypercalcemia but also hypocalcemia. As shown earlier, loss of the CaSR in the setting of loss of either PTH or the parathyroid glands does not "rescue" the double-KO mice from hypoparathyroidism (24, 42), and, as observed here, the C^+P^- and C^-P^- mice have similar degrees of hypocalcemia (6–7 mg/dl). This result has the important and well-recognized implication that hypocalcemia-induced, CaSR-mediated secretion of PTH provides a key, powerful "floor" defending against the development of either acute or prolonged hypocalcemia. Indeed, with adequate levels of vitamin D and normal renal function, only marked, prolonged Ca^{2+} deficiency, as occurs in certain regions of Africa (36), can cause persistent hypocalcemia and, in turn, the associated skeletal lesions of osteomalacia and/or rickets.

An interesting and unanticipated result of our study is that hypercalcemia-induced, CaSR-mediated suppression of PTH is not needed for a robust defense against various hypercalcemic challenges (e.g., increased oral intake of Ca^{2+} , PTH injection/infusion, or phosphate depletion). That is, the C^-P^- mice effectively lack a "ceiling" above which it is difficult to elevate Ca^{2+} , through a hypercalcemic challenge, and, instead, they exhibit marked hypercalcemic responses, particularly those arising from PTH infusion via minipump and phosphate depletion. The serum Ca^{2+} of the C^+P^- mice, in contrast, increases to a level very similar to that of the C^+P^+ mice receiving the same challenge, and it is difficult to elevate their serum Ca^{2+} concentration above 10–11 mg/dl, i.e., the upper limit of normal.

What is the importance of various tissues in the CaSR-mediated defense against hypercalcemia in the C^+P^+ and C^+P^- mice that is lacking in the C^-P^- mice? The kidney likely plays a key role in this regard. C^+P^- and C^+P^+ mice showed marked and similar elevations in urinary Ca^{2+} excretion with increases in Ca^{2+} intake, which appears to provide an important mechanism for maintaining normocalcemia during oral Ca^{2+} loading. Since the responses of these two genotypes to an oral Ca^{2+} load were similar, it appears, at least in mice, that a direct action of Ca^{2+} on renal tubular handling of Ca^{2+} is a dominant mechanism underlying this effect and that suppression of PTH as serum Ca^{2+} rises is not an absolute requirement for upregulating Ca^{2+} excretion. In contrast, there was a right shift in the relationship between serum and urine Ca^{2+} during oral Ca^{2+} loading in C^-P^- mice, which was even more marked during PTH infusion, consistent with available evidence that PTH upregulates the molecular machinery mediating renal Ca^{2+} reabsorption in the distal convoluted tubule (DCT) (43). It is possible that the reduction in serum phosphate observed in the C^-P^- mice during oral Ca^{2+} loading partially overcame the CaSR's hypocalciuric action, as phosphate depletion causes hypercalciuria in CaSR-intact animals and people (4, 25). Thus, the failure of the kidney to upregulate Ca^{2+} excretion in the face of hypercalcemia in the C^-P^- mice is likely an important contributor to their intolerance to oral Ca^{2+} loading and to hypercalcemia induced by PTH infusion.

Where is the most likely site of action of the CaSR in the kidney that is perturbed in the C^-P^- mice? There is a clear precedent for a key role of the cTAL (cortical thick ascending limb) in producing absolute or relative hypocalciuria in pa-

tients with familial hypocalciuric hypercalcemia (FHH) and NSHPT, who are heterozygous and homozygous, respectively, for inactivating CaSR mutations (1). Even in patients with FHH/NSHPT who were aparathyroid, there was persistent, excessively avid renal tubular reabsorption of Ca^{2+} , which was substantially reduced by the loop diuretic ethacrynic acid (1). Further investigations, beyond the scope of this study, will be needed to perform similar studies in the mouse models employed here. The importance of other segments also requires further investigation. The CaSR, like the PTH receptor, is also expressed in the DCT, a site where thiazide diuretics are known to modulate renal tubular calcium reabsorption in a PTH-dependent (3, 20, 32) and -independent manner (35, 37), but we did not examine the CaSR's role in this nephron segment.

We did not directly assess gastrointestinal Ca^{2+} absorption. However, the similar levels of $1,25(\text{OH})_2\text{D}_3$ in the C^+P^- and C^-P^- mice, when receiving both 0% and 2% Ca^{2+} water, suggests that abnormalities in $1,25(\text{OH})_2\text{D}_3$ metabolism and, presumably, in its action on the intestine were not important causes of the intolerance of the C^-P^- mice to oral Ca^{2+} loading. In fact, if anything, the level of $1,25(\text{OH})_2\text{D}_3$ in the C^-P^- mice was lower than that in the C^+P^- mice when both genotypes were receiving 2% Ca^{2+} in the drinking water, although this difference was not statistically significant.

In a similar manner, the levels of $1,25(\text{OH})_2\text{D}_3$ rose equivalently in C^+P^+ , C^+P^- , and C^-P^- mice fed a nominally phosphate-free diet despite the markedly higher level of serum Ca^{2+} that developed in the C^-P^- mice under these conditions. Therefore, a difference in $1,25(\text{OH})_2\text{D}_3$ levels also cannot account for the marked hypercalcemia in the phosphate-depleted C^-P^- mice. We were initially surprised at the failure of $1,25(\text{OH})_2\text{D}_3$ levels to fall in mice maintained on 2% Ca^{2+} water, at least in the C^+P^+ and C^+P^- genotypes, since hypercalcemia generally reduces $1,25(\text{OH})_2\text{D}_3$ levels indirectly by suppressing PTH secretion and directly by inhibiting 1α -hydroxylation in the proximal tubule (4, 46).

There was a near-total abolition of the stimulation of CT by hypercalcemia in the C^-P^- mice, in marked contrast to the C^+P^+ and C^+P^- mice, both of which showed robust increases in serum CT during oral Ca^{2+} loading. The C^-P^- mice exhibited a responsiveness to the hypocalcemic action of exogenous CT that was similar to or greater than that of the C^+P^- mice. The lack of a hypocalcemic action of CT on the C^+P^+ mice presumably resulted from their capacity to mount a sufficient PTH response to forestall the development of hypocalcemia, at least with this dose of CT. In any event, the failure of the C^-P^- mice to mount a CT response to hypercalcemia, rather than impaired responsiveness to their admittedly low endogenous CT levels, likely contributes, along with altered renal Ca^{2+} handling, to their development of hypercalcemia during oral Ca^{2+} loading. In contrast, in the C^+P^+ and C^+P^- mice, hypercalcemia-induced CT secretion and upregulation of renal Ca^{2+} excretion both appear to be important components of their homeostatic ceiling defending against hypercalcemia. From our data, however, we cannot quantify the relative importance of these two mechanisms to the defense against hypercalcemia in mice or, for that matter, in humans.

Changes in urinary DPD and serum osteocalcin levels could not explain the differential responses of the C^+P^- and C^-P^- mice to hypercalcemic challenges. That is, there was neither a

higher level of a bone resorption marker (i.e., DPD) nor a reduced level of a bone formation marker (osteocalcin) in the hypercalcemic $C^{-}P^{-}$ mice compared with the normocalcemic $C^{+}P^{-}$ mice ingesting 2% Ca^{2+} water. This result must be interpreted with caution, however, given the available data suggesting that the CaSR lacking exon 5 in the CaSR KO model utilized here may be hypomorphic with respect to CaSR-mediated actions in cartilage and bone (9). It is of interest that both the $C^{+}P^{+}$ and $C^{+}P^{-}$ mice exhibited a fall in serum OC when changed from 0% to 2% Ca^{2+} drinking water whereas serum OC did not change in the $C^{-}P^{-}$ mice, suggesting a role for the CaSR in OC production/secretion. Of note in this regard, serum OC levels nearly doubled in normal human subjects during induction of hypocalcemia by infusion of EDTA (19), but the relevance of this acute action of hypocalcemia to the more chronic effects of oral Ca^{2+} loading in our studies is unclear.

In summary, the CaSR contributes to the defense against both hypo- and hypercalcemia. The ability of the parathyroid gland, in a CaSR-dependent manner, to increase PTH secretion, PTH gene expression, and parathyroid cellular proliferation in response to hypocalcemia provides a powerful homeostatic defense against hypocalcemia that couples enhanced PTH secretion to its actions on kidney and bone and indirectly, via its stimulation of 1,25(OH) $_2$ D $_3$ synthesis, on intestine. The defense against hypercalcemia, in contrast, at least in mice, does not require CaSR-induced suppression of parathyroid function but instead utilizes CaSR-mediated upregulation of urinary Ca^{2+} excretion and CT secretion and, perhaps, additional mechanisms in bone, intestine, and additional tissues not defined here.

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