

Calcitonin Stimulation of Renal 25-Hydroxyvitamin D-1 α -Hydroxylase Activity in Hypophosphatemic Mice

Evidence That the Regulation of Calcitriol Production Is Not Universally Abnormal in X-linked Hypophosphatemia

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Abstract

Hypophosphatemia (Hyp) mice have defective regulation of 25(OH)D-1 α -hydroxylase activity in response to hypophosphatemia, hypocalcemia, and parathyroid hormone (PTH) administration. However, recent observations support the existence of anatomically distinct, independently regulated renal 1 α -hydroxylase systems in mammalian proximal convoluted and straight tubules. To more completely define the extent of the 1 α -hydroxylase regulatory defect in Hyp-mice, we compared enzyme maximum velocity in normal and mutants after infusion of calcitonin. Upon stimulation, renal 1 α -hydroxylase activity increased to similar levels in normal and Hyp-mouse renal homogenates. Moreover, time-course and dose-dependence studies revealed similar patterns of response in the animal models. Subsequently, we examined whether PTH and calcitonin stimulatory effects on enzyme activity are mediated through different mechanisms. In both animal models administration of PTH and calcitonin increased enzyme activity to levels greater than those obtained after maximal stimulation by either hormone alone, consistent with additive effects. These observations indicate that a calcitonin-sensitive component of 1 α -hydroxylase is not compromised in the X-linked hypophosphatemic syndrome.

Introduction

Abnormal vitamin D metabolism is a component of X-linked hypophosphatemia (XLH),¹ the prototypic vitamin D-resistant rachitic disease in man. Despite chronic hypophosphatemia, which is usually associated with increased 1,25-dihydroxyvitamin D [1,25(OH)₂D] production, normal serum calcitriol levels are present in both affected subjects (1, 2) and in the murine homologue of the human disorder, the hypophosphatemic (Hyp-) mouse (3). Studies in the Hyp-mouse have established that there is aberrant regulation of renal 25(OH)D-1 α -hydroxylase activity, including altered enzyme response to phosphate depletion (3,

4) as well as impaired response to multiple other stimuli including hypocalcemia and parathyroid hormone (PTH) (5-9). While these data suggest a generalized defect of renal 25(OH)D-1 α -hydroxylase in Hyp-mice (and in patients with XLH), recent studies indicate that these observations are insufficient to establish the ubiquity of the derangement. In this regard, Akiba and associates (10) and Kurokawa and associates (11, 12), using microdissected nephron segments, have identified the presence of two distinct and uniquely regulated 25(OH)D-1 α -hydroxylase systems located in the proximal convoluted (PCT) and proximal straight (PST) tubules of the mammalian kidney. To date, evaluation of the renal 25(OH)D-1 α -hydroxylase regulation in Hyp-mice has been confined to stimulants that likely affect enzyme localized in the PCT. Thus, the integrity of enzyme function in the PST has not been examined. Therefore, in the present study we investigated whether the renal 25(OH)D-1 α -hydroxylase of Hyp-mice responds normally to calcitonin in order to more completely define the magnitude of the expressed defect in enzyme regulation. We assessed the 25(OH)D-1 α -hydroxylase in Hyp-mice and age-matched normals using a sensitive *in vitro* assay of murine enzyme activity.

Methods

Animals

Normal C57BL/6J mice were mated with C57BL/6J heterozygous female Hyp-mice as previously described (13). Male and female weanling Hyp-mice obtained from the resultant litters were identified (13) and selected for study at 4-5 wk of age. An equal number of male and female normal littermates were also chosen for investigation. The mice were fed a standard commercial diet containing 0.6% of both calcium and inorganic phosphorus (Teklad Co., Madison, WI) and received deionized water *ad lib.* from the time of weaning until study 4-5 wk later. At this time the mice were exsanguinated and kidneys removed for measurement of renal 25(OH)D-1 α -hydroxylase activity.

Experimental protocols

STIMULATION OF RENAL 25(OH)D-1 α -HYDROXYLASE ACTIVITY IN NORMAL AND HYP-MICE

Continuous infusion of calcitonin. In initial investigations, we examined the effect of calcitonin on renal 25(OH)D-1 α -hydroxylase activity in normal and Hyp-mice. To control the uniformity of the applied stimulus, we used surgically implantable Alzet osmotic minipumps (model 2001; Alza Corp., Palo Alto, CA) for continuous subcutaneous infusion of calcitonin. Human synthetic calcitonin (3,000 IU/ μ l, Sigma Chemical Co., St. Louis, MO) was added to physiologic saline (0.9% NaCl) and minipumps filled with the calcitonin solution (0.5 IU/ μ l). After sodium pentobarbital anesthesia (65 mg/kg *i.p.*), the minipumps were implanted subcutaneously in the normal and Hyp-mice through a small skin incision in the dorsal lumbar area. The incision was closed with a wound clip. Controls were not anesthetized or sham operated, since these procedures do not alter renal 25(OH)D-1 α -hydroxylase activity in normal or Hyp-mice (data not shown). After 24 h the mice were exsanguinated, dis-

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1. *Abbreviations used in this paper:* Hyp-, hypophosphatemic; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; PCT, proximal convoluted tubules; PST, proximal straight tubules; PTH, parathyroid hormone; XLH, X-linked hypophosphatemia.

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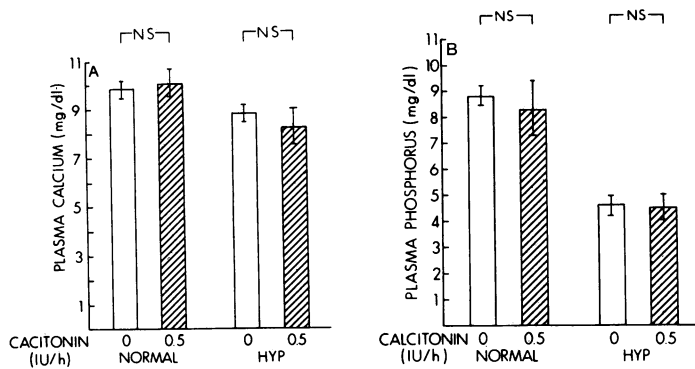


Figure 1. (A) Plasma calcium and (B) inorganic phosphorus concentrations of normal and Hyp-mice before and 24 h after continuous subcutaneous administration of 0.5 IU/h synthetic human calcitonin. In each mouse model the significance of the differences between pre- and post-infusion plasma calcium and phosphorus concentrations was assessed by the paired *t* test. Each bar represents the mean \pm SE of at least five separate determinations.

patched, and the kidneys excised for measurement of 25(OH)D-1 α -hydroxylase activity. Plasma calcium, inorganic phosphorus, and immunoreactive PTH were determined for all groups.

The time-course and dose dependency of 25(OH)D-1 α -hydroxylase response to calcitonin infusion were evaluated in the normal and Hyp-mouse models. In the former experiment, age- and sex-matched normal and Hyp-mice were assigned to either pump-infused or control groups. Infused mice received a dose of 0.5 IU/h calcitonin, and pumps were implanted 3, 6, 12, 18, 24, 48, and 72 h before exsanguination. Dose responses to calcitonin were examined in normal and Hyp-mice implanted with pumps infusing 0.1, 0.375, 0.5, 0.75, 1.0, or 1.25 IU/h calcitonin 24 h before study.

Additive effects of PTH and calcitonin. In subsequent studies we examined whether PTH and calcitonin effects on renal 25(OH)D-1 α -hydroxylase activity are mediated by different mechanisms and/or occur in separate and distinct nephron segments. We evaluated whether simultaneous administration of these hormones (at doses that elicit a maximal enzyme response) results in an additive stimulation of the 25(OH)D-1 α -hydroxylase. Age- and sex-matched normal and Hyp-mice served as controls or were subjected to subcutaneous infusion with calcitonin, PTH, or calcitonin and PTH. Calcitonin was administered via an Alzet minipump (Alza Corp.) at a rate of 1.25 IU/h for 24 h before study. Bovine parathyroid extract (138 IU/mg, Sigma Chemical Co.) dissolved in physiologic saline (0.9% NaCl) containing 20 mg/ml cysteine hydrochloride was administered similarly at 1.0 IU/h. Animals receiving both hormones were implanted through separate skin incisions with two minipumps containing PTH and calcitonin, respectively.

Analytical methodology

We measured the maximum velocity of 25(OH)D-1 α -hydroxylase in kidney homogenates by our previously described method (14). Plasma calcium was determined by atomic absorption spectrophotometry (272 Spectrophotometer; Perkin Elmer Corp., Instrument Div., Norwalk, CT) and plasma inorganic phosphorus by a colorimetric technique (15). We assayed plasma PTH using a commercially available radioimmunoassay kit (Immunonuclear Corp., Stillwater, MN). The assay is a disequilibrium procedure in which an antibody developed against the 44–68 region of human PTH is used. The antibody has a well characterized cross-reactivity with a reference standard derived from rat parathyroid gland homogenates. The assay also cross-reacts well with mouse PTH, allowing measurement of normal, decreased and elevated levels. The limit of detection for the assay is 15 pmol/liter and the interassay coefficient of variation is 9.5%.

Statistical methods

Data are expressed as mean \pm SE. We evaluated the data statistically using paired *t* testing and regression analysis (16).

Materials

Dr. Milan Uskokovic, Hoffmann-La Roche, Nutley, NJ, kindly provided authentic 25(OH)D₃ and 1,25(OH)₂D₃ for our studies. We purchased [³H]1,25(OH)₂D₃ (92 Ci/mmol) from Amersham Searle Corp., Arlington Heights, IL.

Results

Stimulation of renal 25(OH)D-1 α -hydroxylase activity in normal and Hyp-mice

Continuous infusion of calcitonin. Administration of calcitonin (0.5 IU/h, subcutaneously) to normal and Hyp-mice did not result in significant changes of either plasma calcium or phosphorus concentrations after 24 h (Fig. 1). Similarly, no differences were observed in plasma PTH levels either before or after 24 h of calcitonin infusion (Fig. 2). In contrast, both normal and Hyp-mice responded to the standard calcitonin stimulus with a significant enhancement of renal 25(OH)D-1 α -hydroxylase activity. Moreover, the level of enzyme activity achieved after hormonal stimulation was identical in both animal groups (Fig. 3).

Normal enzyme responsiveness in the Hyp-mouse was confirmed by demonstrating a time-course of calcitonin stimulation in affected animals which was similar to that in normals. Enzyme activity in response to 0.5 IU/h calcitonin at various times ranging from 0 to 72 h of infusion displayed a linear and parallel increase in both normal and Hyp-mice that plateaued between 18 and 24 h. In addition, a precipitous fall in activity occurred by 48 h of hormone administration (Fig. 4).

In addition, Hyp-mice sustained no phase shift in response to calcitonin stimulation either to the right or left of that demonstrated in normal animals. Dose-response studies over 24 h revealed that, in response to graduated amounts (0–1.25 IU/h) of administered calcitonin, normal and Hyp-mice exhibited indistinguishable curvilinear increases of enzyme activity, achieving a plateau at 1.0 IU/h (Fig. 5).

Additive effects of calcitonin and PTH. In subsequent studies, we sought to determine whether the simultaneous administration of calcitonin and PTH had additive effects on renal 25(OH)D-1 α -hydroxylase activity in both normal and Hyp-mice. In response to PTH infusion (1.0 IU/h), normal mice exhibited an eightfold enhancement of enzyme activity, while mutants dis-

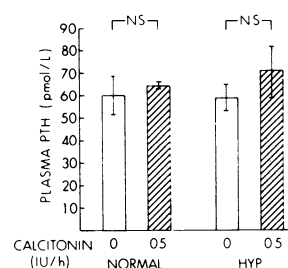


Figure 2. Plasma PTH concentration of normal and Hyp-mice before and 24 h after continuous subcutaneous administration of 0.5 IU/h synthetic human calcitonin. In each mouse model the significance of the difference between the pre- and post-infusion plasma PTH concentration was assessed by the paired *t* test. Each bar represents the mean \pm SE of four individual determinations.

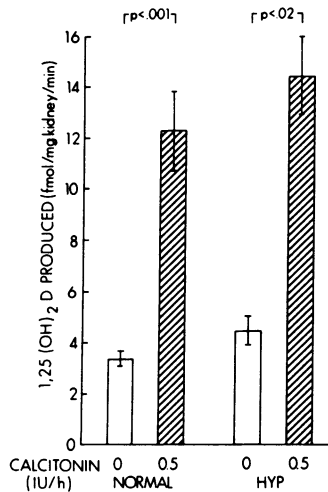


Figure 3. 25-hydroxyvitamin D-1 α -hydroxylase activity of normal and Hyp-mice after continuous subcutaneous infusion of 0.5 IU/h synthetic human calcitonin. All samples were incubated at 37°C with 80 μ M 25(OH) $_2$ D $_3$ provided as substrate. Data were analyzed by two-way analysis of variance and the Bonferroni multiple range comparison test. Each bar represents the mean \pm SE from at least six individual experiments.

played a significantly lesser fourfold increment, similar to previously reported observations (8). In contrast, a maximal stimulatory dose of calcitonin (1.25 IU/h) resulted in an identical increase of 25(OH)D-1 α -hydroxylase activity in both the normals and the mutants, as reported above. In both animal models, however, the simultaneous administration of both hormones increased enzyme activity to levels greater than those achieved by either PTH or calcitonin stimulation alone. This augmentation of enzyme activity is consistent with additive effects of the hormones on renal 25(OH)D-1 α -hydroxylase (Fig. 6).

Discussion

A role of abnormal vitamin D metabolism in the pathogenesis of XLH has long been suspected. Direct assay of mammalian renal 25(OH)D-1 α -hydroxylase has confirmed the existence of defective regulation of 1,25(OH) $_2$ D synthesis in the Hyp-mouse model of XLH. Initially, Lobaugh and Drezner (4) reported an inadequate response of renal 25(OH)D-1 α -hydroxylase activity to hypophosphatemia in Hyp-mice. Subsequently, several investigators further characterized the impairment of 1,25(OH) $_2$ D production in the mutant mice. Nesbitt et al. (8) demonstrated that renal 25(OH)D-1 α -hydroxylase activity in Hyp-mice does not increase normally in response to stimulation by calcium

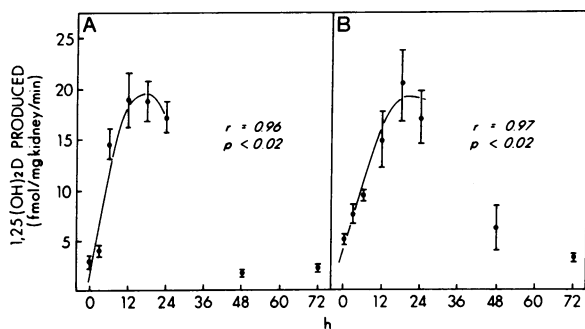


Figure 4. Effect of infusion time on 1,25(OH) $_2$ D $_3$ produced by normal and Hyp-mouse kidney homogenates after stimulation with 0.5 IU/h synthetic human calcitonin. Each point represents the mean \pm SE of determinations obtained from four individual experiments. Each reaction vessel contained 100 mg of kidney and was incubated at 37°C with 80 μ M 25(OH) $_2$ D $_3$ provided as substrate. Regression models were used to fit the data obtained.

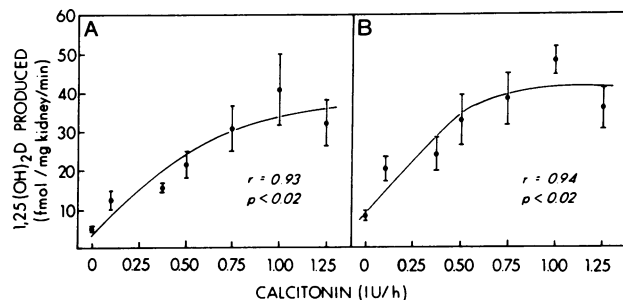


Figure 5. Effect of calcitonin dose on 1,25(OH) $_2$ D $_3$ produced by normal and Hyp-mouse renal homogenates after stimulation with 0.5 IU/h synthetic human calcitonin. Each reaction vessel contained 100 mg of kidney and was incubated at 37°C with 80 μ M 25(OH) $_2$ D $_3$ provided as substrate. Regression models were used to fit the data obtained. Each point shown represents the mean \pm SE from at least four individual experiments.

deficiency and/or PTH. Similarly, Tenenhouse (7) reported that Hyp-mice maintained for 6 wk on a rachitogenic diet exhibit significantly lesser enzyme activity than normal mice fed the same diet. These data provide evidence that the defect in 25(OH)D-1 α -hydroxylase activity in Hyp-mice does not represent altered response to hypophosphatemia alone but is indicative of a generalized regulatory anomaly.

In contrast, the results of the present study suggest that the expressed abnormality of enzyme function is, in fact, incomplete

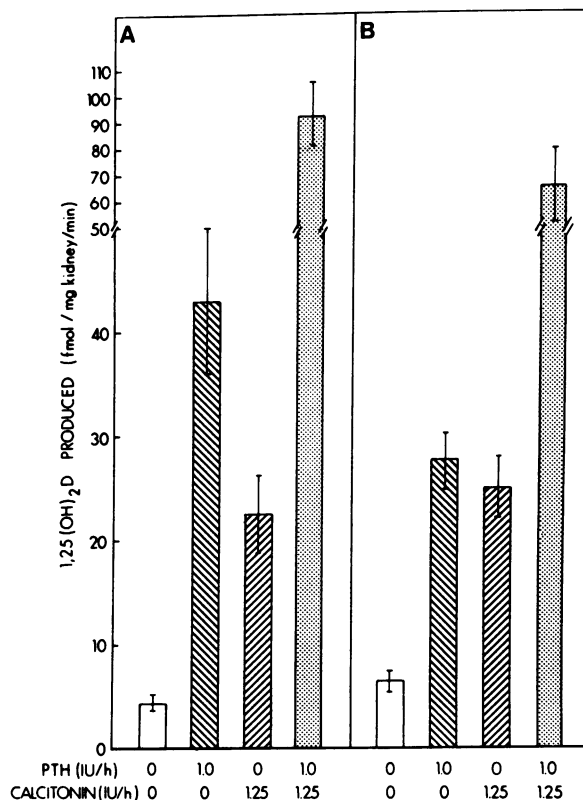


Figure 6. 25-hydroxyvitamin D-1 α -hydroxylase activity of (A) normal and (B) Hyp-mice after continuous subcutaneous administration of bovine PTH and/or synthetic human calcitonin in the doses indicated. All samples were incubated for 20 min at 37°C with 80 μ M 25(OH) $_2$ D $_3$ provided as substrate. Each bar represents the mean \pm SE of at least three individual determinations.

and uniquely limited. After calcitonin infusion (1.25 IU/h) for 24 h, renal homogenates from normal and Hyp-mice exhibited an equivalent increase in enzyme activity. This apparent normal increment of 25(OH)D-1 α -hydroxylase observed in Hyp-mice is contrary to the muted stimulatory effects of calcium deficiency and/or PTH on enzyme activity.

Moreover, examination of the time-course and dose dependency of the calcitonin infusion in normal and Hyp-mice confirms the normal responsiveness to this hormonal stimulus. In kidneys from both normals and mutants the capacity for calcitonin to enhance 25(OH)D-1 α -hydroxylase plateaus at a dose of 1.0 IU/h. In addition, the absolute level of activity achieved in the Hyp-mouse kidney at this dose was indistinguishable from that in normals. Further, in both normal and Hyp-mice, 25(OH)D-1 α -hydroxylase activity reached a peak by 18–24 h after infusion of 1.25 IU/h calcitonin and declined thereafter. The maximal level of enzyme activity achieved was identical in both groups. The loss of responsiveness observed after 24 h may be a function of several unrelated mechanisms, including down-regulation of the calcitonin receptor or product inhibition of the enzyme by increased levels of 1,25(OH)₂D.

The mechanism underlying the variably expressed abnormality of renal 25(OH)D-1 α -hydroxylase activity in Hyp-mice is not immediately apparent. However, the additive effects of calcitonin and PTH on enzyme function indicate that these hormones modulate the enzyme via different mechanisms in the same cells or via similar or different mechanisms in distinctly different cell systems. In this regard, a series of previous studies (12, 17, 18) has established that the effects of PTH on 25(OH)D-1 α -hydroxylase are mediated by cyclic AMP, while those of calcitonin are independent of adenylate cyclase activation (11, 19, 20). Thus, the defective regulation of enzyme function in the Hyp-mouse appears to be confined to the PTH-adenylate cyclase component of activation, while the calcitonin responsive pathway remains unaffected.

Moreover, it is likely that PTH and calcitonin affect enzyme activity not only by different mechanisms but in different cell populations. Recent studies of Kawashima et al. (11, 12, 20) and Akiba et al. (10) have established the presence of two distinct 25(OH)D-1 α -hydroxylase systems in the mammalian kidney. Using microdissected nephron segments from rat and fetal rabbit kidney, these investigators localized enzyme activity in the PCT and PST. In addition, Kawashima et al. (20) demonstrated that the 25(OH)D-1 α -hydroxylase in the PCT is regulated by PTH and (probably) phosphate, but not calcitonin, while that in the PST is stimulated by calcitonin (via an unknown mechanism). We suggest that the abnormal PTH and phosphate-regulated enzyme activity in Hyp-mice may be localized in the PCT. In contrast, the normally calcitonin-responsive 25(OH)D-1 α -hydroxylase is likely in the PST. Further data supporting this possibility derives from previous studies demonstrating impaired adenylate cyclase response to PTH in the PCT of Hyp-mice (21). In addition, since defective phosphate transport in the Hyp-mice undoubtedly occurs in the PCT (22–24), the primary site of phosphate transport (25), it would not be surprising if this were the site of the abnormal vitamin D metabolism. Indeed, aberrant response of the PCT 25(OH)D-1 α -hydroxylase may result from abnormal phosphate transport and consequent changes in the intracellular milieu. Therefore, the defective enzyme activity in the Hyp-mice (and patients with XLH) may not be a genetically determined abnormality of 25(OH)D-1 α -hydroxylase structure/function, but rather an acquired defect

secondary to a seemingly unrelated abnormality, impaired phosphate transport.

The stimulatory effects of calcitonin on 25(OH)D-1 α -hydroxylase activity that we observed are consistent with previous animal studies (26). In contrast, in vitro evaluation of calcitonin effects on enzyme function have been controversial. Although Larkins et al. (27) reported stimulation of enzyme activity in isolated renal tubules from the kidneys of vitamin D-deficient chicks, other investigators (28, 29) have found no effect, or actual inhibition, of 25(OH)D-1 α -hydroxylase in isolated tubules and/or renal cell cultures. The reasons underlying these disparities remain unclear. Lorenc et al. (28) have suggested that the failure to increase activity in vitro results from calcitonin action being mediated in vivo by PTH, but this seems unlikely. Indeed, in our experiments we demonstrated no significant changes in plasma calcium or PTH after calcitonin stimulation. Moreover, the stimulation of 1-OHase after simultaneous administration of PTH and calcitonin to levels significantly greater than those obtained with maximal doses of either hormone alone is consistent with the notion that the effects of the hormones are additive rather than interdependent. Thus, the disparate results may represent species differences, variations within the cell populations of the experimental models, or the existence of unrecognized conditions in vivo for calcitonin effects to become apparent.

In contrast to our studies, Fukase et al. (6) have reported that salmon calcitonin caused a significant increase in 25(OH)D-1 α -hydroxylase in primary tissue cultures of renal cortical cells from normal mice, whereas enzyme activity in matched cell populations from Hyp-mice did not change. These studies are complicated by the difficulties inherent to the study of enzyme activity in an in vitro system as noted above. In addition, interpretation of these data assumes that the distribution of renal cell types is similar in the cultures from normal and Hyp-mice. To support this assumption Fukase et al. (6) demonstrated that cyclic AMP accumulation in response to PTH, calcitonin, and vasopressin stimulation was similar in cultures from both genotypes. However, the magnitude of PTH- and calcitonin-dependent adenylate cyclase activity is different in the renal tubules of normal and Hyp-mice. Thus, the presence of equal increments of cyclic AMP in cultures from normal and Hyp-mice indicate that the distribution of cell types may not have been identical. Consequently, we cannot adequately compare the data obtained in the present study with that reported by Fukase et al. (6).

We believe that our measurements of renal 25(OH)D-1 α -hydroxylase provide new and important data necessary to a complete understanding of the pathophysiology underlying XLH. The present findings, together with the results of our earlier reports (4, 8), support the hypothesis that enzyme activity in the Hyp-mouse model of XLH is abnormally regulated by factors that influence enzyme function in the PCT. In contrast, enzyme function in the PST is normally modulated. We postulate that the abnormal vitamin D regulation in the Hyp-mouse (and in patients with XLH) likely results from alterations in the intracellular milieu of the proximal convoluted tubular cell due to alterations in phosphate transport, the primary genetic abnormality of the disease.

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