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Parathyroid gland calcium receptor mRNA levels are unaffected by chronic renal insufficiency or low dietary calcium in rats

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Extracellular ionized calcium (Ca^{2+}) is the primary physiological regulator of parathyroid hormone (PTH) secretion and the G protein-coupled receptor (CaR) that mediates this response has been cloned from bovine and human parathyroid glands. The Ca^{2+} set-point for the regulation of PTH secretion is right-shifted in primary hyperparathyroidism (1°HPT), but whether there is a similar shift in 2°HPT is unclear. Additionally, the molecular defects associated with such changes in the set-point remain uncharacterized. These experiments were designed to determine (1) if changes in set-point occur in rats with 2°HPT induced by chronic renal insufficiency (CRI) or dietary Ca deficiency, and (2) whether any changes in set-point are mirrored by changes in steady-state mRNA levels for the parathyroid CaR. CaR mRNA levels were quantified in pairs of glands from individual rats using a solution hybridization assay. Blood urea nitrogen and PTH levels were ~4-fold higher in rats with CRI induced by 5/6 nephrectomy 7 weeks earlier. Rats with CRI were also significantly hypocalcemic and hyperphosphatemic. The set-point was unchanged in CRI rats and CaR mRNA levels were also unaffected. Normal rats fed a 0.02% Ca diet for 6 weeks were markedly hypocalcemic, and had 10- and 15-fold increases in plasma PTH and 1,25-dihydroxyvitamin D_3 levels, respectively. Technical problems prevented assessment of the set-point in these animals, but parathyroid gland CaR mRNA levels were identical in both dietary groups. Thus, neither alterations in mRNA levels for the CaR nor changes in the set-point play demonstrable roles in the pathogenesis of 2°HPT in these models.

Keywords: calcium receptor; parathyroid glands; secondary hyperparathyroidism; chronic renal insufficiency; calcium deficiency; rat; solution hybridization assay

Introduction

The concentration of ionized calcium (Ca^{2+}) in the extracellular fluid is the primary physiological regulator of parathyroid hormone (PTH) secretion and a steep inverse sigmoidal relationship exists between plasma Ca^{2+} levels and PTH secretion (Mayer & Hurst 1978; Brown, 1991; Fox, 1991). The Ca^{2+} receptor (CaR) that mediates this extracellular Ca^{2+}-sensing property has been cloned from bovine and human parathyroid glands (Brown *et al.*, 1993; Garrett *et al.*, 1995), and more recently from rat kidney (Riccardi *et al.*, 1995). It is predicted to be an ~120 kDa glycosylated protein with a large extracellular domain coupled to a seven transmembrane spanning region similar to those in other G protein-coupled receptors.

The 'set-point' for regulation of PTH secretion, an index of parathyroid gland sensitivity to extracellular Ca^{2+}, is defined as the Ca^{2+} concentration which elicits a 50% inhibition of the maximum PTH secretion rate (Brown (1991). There is evidence for alterations in this set point in hyperparathyroid states. For example, the set-point is right-shifted both *in vivo* and in dispersed parathyroid cells from patients with primary hyperparathyroidism (1°HPT) (Brown *et al.*, 1979; D'Amour *et al.*, 1994). Increased PTH secretion in 1°HPT results in hypercalcemia which, because of a putative defect in the Ca^{2+}-sensing mechanism, fails to inhibit hormone secretion appropriately. Whether the set-point is affected in 2°HPT is less well understood. The set-point was right-shifted when assessed in dispersed parathyroid cells from patients with chronic renal insufficiency (CRI) undergoing parathyroidectomy for severe 2°HPT (Brown *et al.*, 1982). In contrast, more recent studies in 2°HPT associated with CRI (Ramirez *et al.*, 1993; Goodman *et al.*, 1994; St. John *et al.*, 1994; Sanchez *et al.*, 1994), dietary calcium and vitamin D deficiency (Cloutier *et al.*, 1992), or advancing age (Fox, 1991; Udén *et al.*, 1992; Ledger *et al.*, 1994) have not consistently identified a defect in the parathyroid gland sensitivity to Ca^{2+}; increases, decreases and no change in set-point have been reported. The controversy surrounding whether the set-point is altered in 2°HPT may result from the severity of the disorder, its pathology and pathogenesis or, at least in some of the earlier studies, the use of heterologous and insensitive immunoassays for PTH that were not specific for the intact peptide.

The molecular mechanisms responsible for changes in parathyroid gland sensitivity to Ca^{2+} in both 1° and 2°HPT remain unknown, although there is currently no evidence for mutations in the CaR gene in parathyroid adenomas (Capuano *et al.*, 1994). In contrast, point mutations of the CaR gene sequence in familial benign hypocalciuric hypercalcemia can result in a right-shift in the set-point for PTH release (Marx *et al.*, 1985; Khosla *et al.*, 1993; Pollak *et al.*, 1993; Heath 1994; Heath *et al.*, 1994; Pearce *et al.*, 1994). Based on these observations we formed the hypothesis that not only mutations in the CaR gene, but also changes in the level of expression of the normal CaR gene may alter the sensitivity of the parathyroid gland to extracellular Ca^{2+}. Supporting this hypothesis is the observation that culturing of bovine parathyroid cells results in a progressive loss of sensitivity to Ca^{2+} and a right-shifted set-point that is associated with a rapid reduction in the expression of both CaR mRNA and protein (LeBoff *et al.*, 1985; Mithal *et al.*, 1995). The suggestion that reduced levels of functional CaR protein may be associated with an elevated set-point is consistent with the observation that a variety of G-protein coupled receptors can undergo downregulation which is associated with a corresponding decrease in functional responses (Galper *et al.*, 1982; Findlay & Martin, 1984; Teitelbaum *et al.*, 1986).

These experiments were designed to determine whether the set-point for PTH release was affected in two different rat models of 2°HPT. The models used were mild-to-moderate 2°HPT resulting from CRI and a more-severe 2°HPT, a result of prolonged dietary Ca deficiency. We also sought to determine if changes in set-point were reflected by corresponding changes in the steady-state levels of mRNA for the CaR in the parathyroid glands.

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Results

Effects of chronic renal insufficiency on set-point and parathyroid gland CaR gene expression

Blood urea nitrogen (BUN) and plasma PTH levels were elevated 3.5- and 4.0-fold, respectively, in rats subjected to a 5/6 nephrectomy seven weeks before study, confirming the successful induction of CRI and the development of 2°HPT (Figure 1). Plasma Ca^{2+} levels were slightly, but significantly reduced and plasma phosphate levels were slightly, but significantly elevated in rats with CRI. Plasma 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] levels, although lower in rats with CRI, were not significantly different from those in sham-operated controls (Figure 1).

The mean plasma PTH *vs.* Ca^{2+} levels curves for the sham-operated and CRI rats in the set-point experiments are shown in Figure 2. The calculated mean maximum PTH levels were significantly ($P<0.04$) higher in rats with CRI (117 ± 10 *vs.* 83 ± 13 pg/ml), whereas the calculated mean minimum PTH levels, although 51% higher in CRI rats, were not significantly different (5.8 ± 1.2 *vs.* 3.8 ± 0.5 pg/ml). Similarly, there were no significant differences in the slopes of the curves in the sham-operated (22 ± 2) and CRI rats (18 ± 6), or in the set-points (Figure 3). The levels of mRNA for the CaR in parathyroid glands were also not significantly different between sham-operated and CRI rats (Figure 3). Regression analysis of results from all animals in which both set-point and CaR mRNA levels were obtained showed that there was no significant correlation between parathyroid gland CaR mRNA levels and the set-point ($r = -0.44$, $n = 7$).

Effects of dietary Ca deficiency on parathyroid gland CaR gene expression

Feeding rats the 0.02% Ca diet for 6 weeks induced a marked Ca deficiency and severe 2°HPT. The resultant hypocalcemia was associated with 10.3- and 15.0-fold increases in the plasma levels of PTH and $1,25(OH)_2D_3$, respectively. Plasma phosphate levels were also significantly elevated in Ca-deficient rats (Figure 4).

As described in the Methods section, technical problems prevented assessment of the set-point in rats fed the semi-synthetic Ca-replete and Ca-deficient diets. However, the levels of the mRNA for the CaR were identical in parathyroid glands of rats fed the normal- and low-Ca diets (Figure 4).

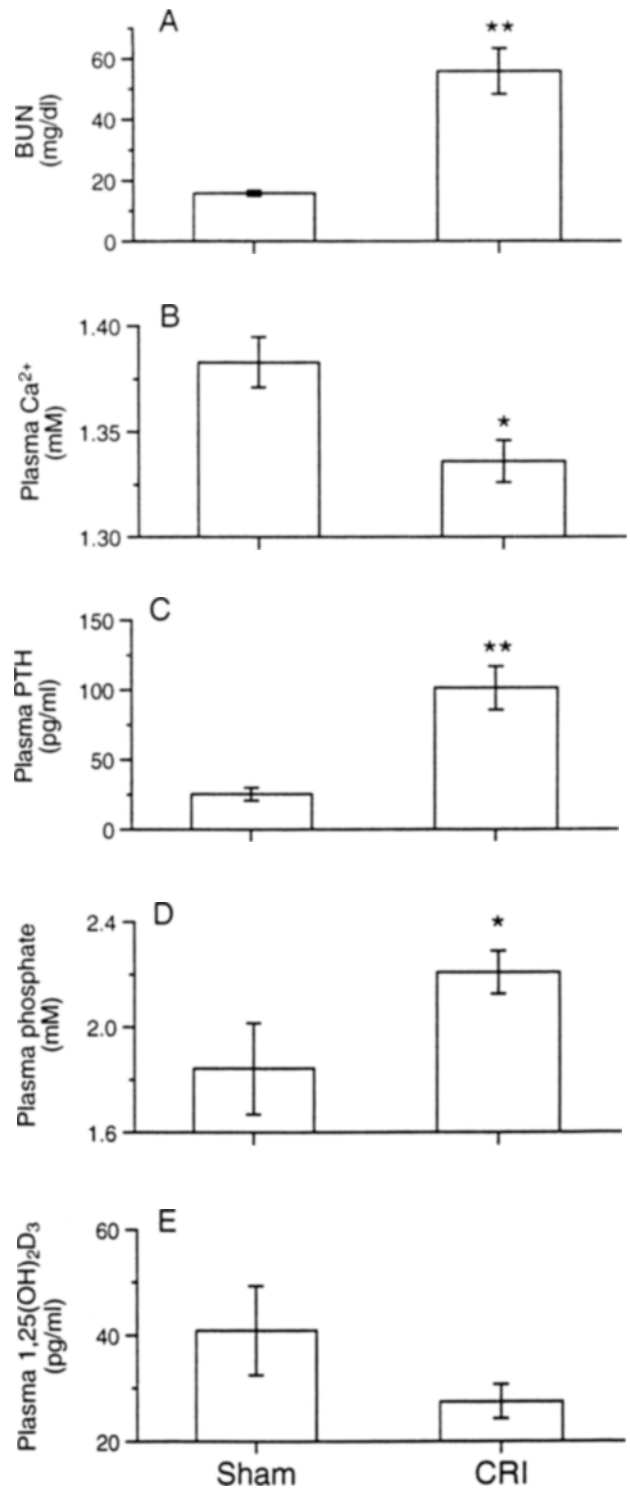

Figure 1 Blood urea nitrogen (BUN), and plasma Ca^{2+}, PTH, phosphate and $1,25(OH)_2D_3$ levels in normal rats subjected to a sham-operation and in rats with CRI induced by a 5/6 nephrectomy 7 weeks prior to study. Values are means ± S.E. ($n = 6-13$/group). *$P<0.05$; **$P<0.01$: significance of difference from sham-operated controls

Discussion

These studies have shown that the levels of mRNA for the CaR in parathyroid glands of rats are not affected in mild-to-moderate 2°HPT resulting from CRI induced by 5/6 nephrectomy or in moderate-to-severe 2°HPT resulting from prolonged dietary Ca deficiency. Thus, alterations in mRNA levels for the CaR play no demonstrable role at this stage in the development of 2°HPT in either of these models. Moreover, the set-point for regulation of PTH secretion by plasma Ca^{2+} was unchanged in rats with CRI. Taken together, these observations indicate that the elevated plasma PTH levels in these models of CRI and dietary Ca deficiency do not result from a change in the sensitivity of the parathyroid cell to extracellular Ca^{2+} and/or by changes in expression of the CaR gene.

We have shown previously that steady-state levels of mRNA for the CaR in parathyroid glands and kidney of vitamin D-deficient rats, another model of 2°HPT, are also unaffected by chronic changes in the plasma levels of Ca^{2+} or $1,25(OH)_2D_3$ that span the pathophysiological ranges (Rogers *et al.*, 1995). The results of those and the current studies provide strong evidence that steady-state CaR mRNA levels in the parathyroid glands of rats are not subject to significant chronic regulation by factors that have a profound influence on parathyroid gland function. Because the solution hybridization assay has sufficient power to detect changes of ~30%, it remains possible that small changes in parathyroid gland CaR mRNA levels that we are unable to detect do occur in models of 2°HPT. A preliminary report by Zhong *et al.* (1994) also described no effect of dietary hyperparathyroidism on parathyroid gland CaR mRNA levels in

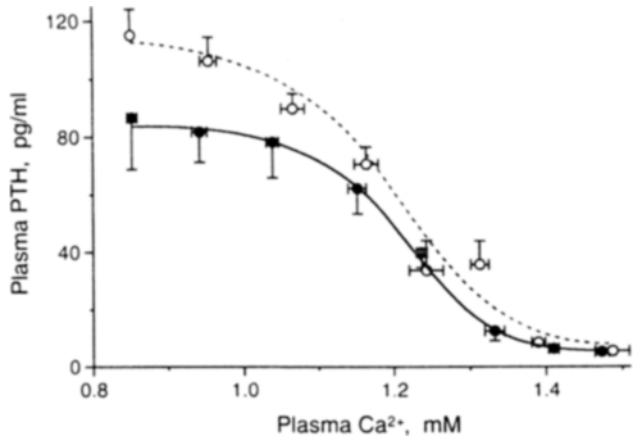

Figure 2 Mean plasma Ca^{2+} *vs* PTH curves during the set-point experiments in sham-operated rats (solid line) and in rats with CRI induced by a 5/6 nephrectomy six weeks before study (dotted line). Values are means ± S.E. ($n = 5-6$/group)

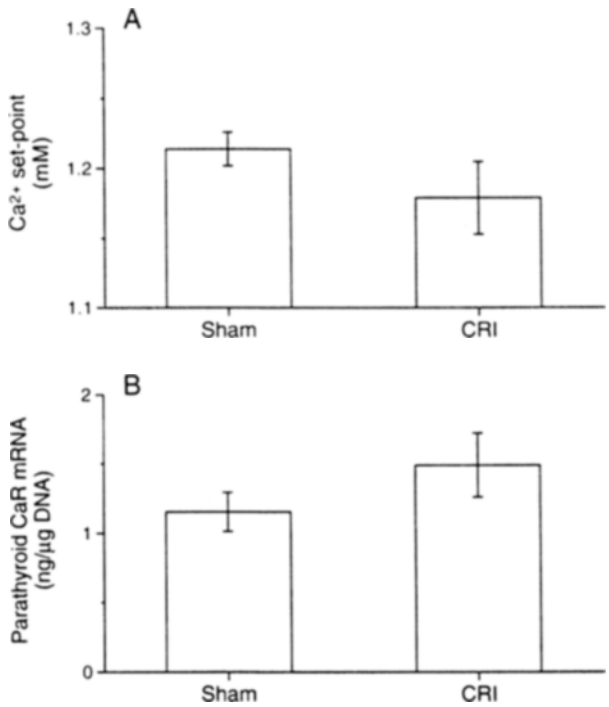

Figure 3 The plasma Ca^{2+} set-point for PTH release (panel A) and CaR mRNA levels in the parathyroid glands (panel B) in normal sham-operated rats and in rats with CRI induced by a 5/6 nephrectomy 6 to 7 weeks prior to study. Values are means ± S.E. ($n = 5-6$/group)

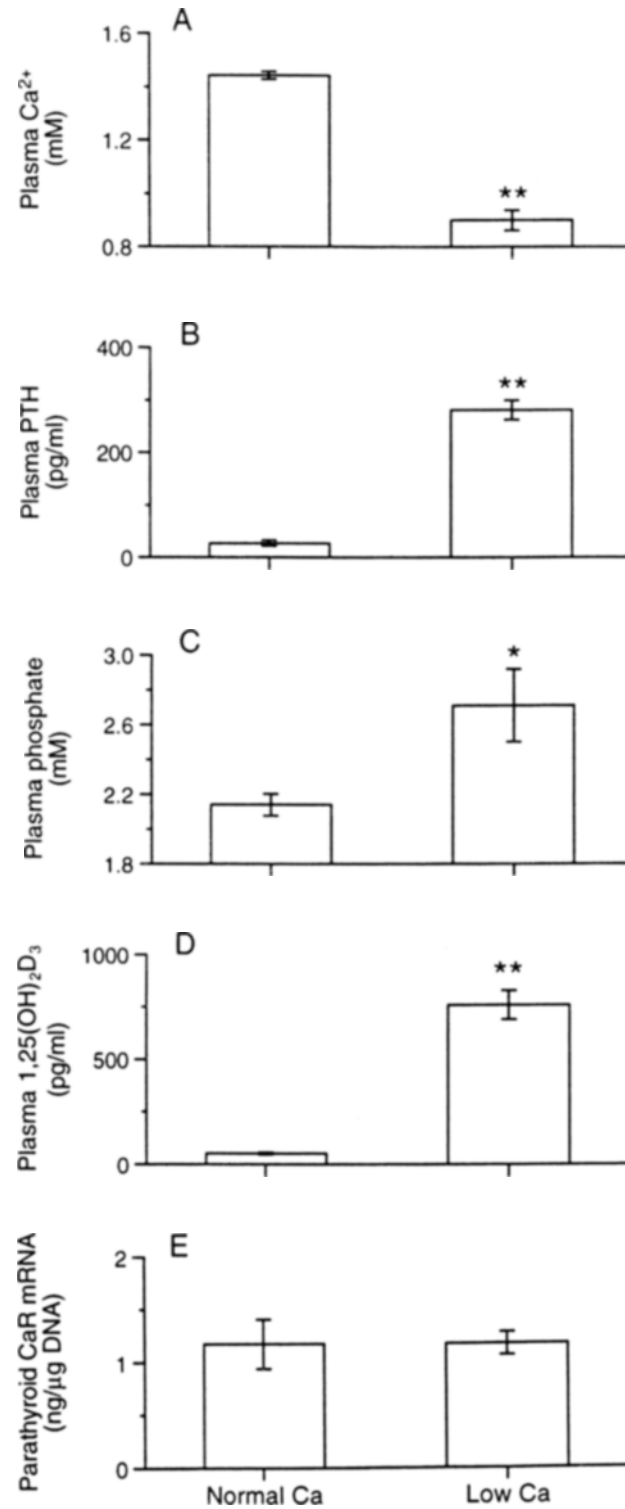

Figure 4 Plasma Ca^{2+}, PTH, phosphate and $1,25(OH)_2D_3$ levels and parathyroid gland CaR mRNA levels in rats fed 0.8% Ca (normal Ca) or 0.02% Ca (low Ca) semi-synthetic diets for 6 weeks prior to study. Values are means ± S.E. ($n = 7-12$/group). $^*P < 0.05$; $^{**}P < 0.01$: significance of difference from normal Ca controls

rats. However, these authors did report that the acute administration of large doses of $1,25(OH)_2D_3$ can result in small, but significant, increases in CaR mRNA levels in the parathyroid glands. Thus, while $1,25(OH)_2D_3$ injections may be capable of increasing CaR mRNA levels acutely, it appears that more physiological, chronic changes in plasma $1,25(OH)_2D_3$ levels induced by $1,25(OH)_2D_3$ infusions (Rogers *et al.*, 1995) or, in these experiments and in those by Zhong *et al.* (1994), by dietary Ca deficiency, do not affect steady-state CaR mRNA levels. Preliminary analysis of the first 2 kb upstream region of the human CaR gene has not revealed any known regulatory motifs such as those associated $1,25(OH)_2D_3$ regulation of the PTH gene (Okazaki *et al.*, 1988; Capuano *et al.*, 1994). It is possible that $1,25(OH)_2D_3$ regulatory elements are located >2 kb upstream or that $1,25(OH)_2D_3$ has indirect effects on CaR transcription by regulating expression of other transcription factors; alternatively, acute increases in CaR mRNA levels elicited by $1,25(OH)_2D_3$ injections could result from changes in mRNA stability.

The plasma Ca^{2+} set-point for PTH release was not affected in rats with CRI confirming the results of most *in vivo* studies in humans with reduced renal function (Ramirez

et al., 1993; Goodman *et al.*, 1994; St. John *et al.*, 1994; Sanchez *et al.*, 1994). Because of the technical problems described in the Methods, it was not possible to measure the set-point in Ca-deficient rats so we do not know if parathyroid gland Ca^{2+} sensitivity is affected in this more-severe form of 2°HPT. Cloutier *et al.*, (1992) have reported a decreased set-point in dogs during the development of dietary Ca and vitamin D deficiency. However, as discussed above, parathyroid gland CaR mRNA levels were unaffected in the Ca-deficient rat model. An increase in set-point has been observed *in vitro* in dispersed parathyroid cells from a patient with severe 2°HPT (Brown *et al.*, 1982), but this may be due to tissue pathology. For example, $1,25(OH)_2D_3$ receptor (VDR) levels are substantially reduced in parathyroid tissue showing nodular hyperplasia (thought to represent a more-advanced stage) than in diffuse hyperplasia (Fukada *et al.*, 1993). This reduction in VDR levels in parathyroid glands may account, in part, for the resistance of parathyroid cells to $1,25(OH)_2D_3$ in uremic hyperparathyroidism (Fukagawa *et al.*, 1991). It is possible that the CaR is also expressed at a lower level in nodular hyperplastic tissue and this, at least in part, may account for a right-shift in the set-point and a resistance to reduction of PTH levels by elevated extracellular Ca^{2+}. Although histological examination was not performed it is unlikely that nodular hyperplasia is present in the rats used in these experiments because the stimulation to the parathyroid glands was relatively mild and of short duration. Additionally, it remains to be determined if parathyroid gland CaR mRNA levels are altered in adenomatous parathyroid glands in which a right-shift in set-point is more consistently observed (Brown *et al.*, 1979; D'Amour *et al.*, 1994).

Materials and methods

Animals and diets

Normal male Sprague-Dawley rats (Harlan Sprague Dawley, Madison, WI) were used in these studies. They were housed in hanging wire cages and permitted unrestricted access to deionized water and food. The rats in which the effects of CRI on CaR gene expression were to be tested (*Experiment 1*) weighed ~250 g upon receipt and were fed commercial rodent chow (Purina no. 5001), which contains 0.95% calcium, 0.67% phosphorus, 23% protein and 4.5 I.U. vitamin D/g. The rats in which the effects of Ca-deficiency were to be tested (*Experiment 2*) weighed ~100 g. From the time of receipt they were fed a semi-synthetic diet (Teklad, Madison, WI) which contained 0.5% phosphorus and 2.2 I.U. vitamin D_3/g and either 0.8% Ca (TD87092; normal-Ca group) or 0.02% Ca (TD88050; low-Ca group). All experimental procedures were approved by the NPS Institutional Animal Care and Use Committee.

Experiment 1. *Regulation of set-point and CaR gene expression in chronic renal insufficiency*

The rats were anesthetized with a combination of ketamine (90 mg/kg) and xylazine (7 mg/kg) injected intramuscularly. CRI was induced by a one-stage subtotal (5/6) nephrectomy which involved removal of one kidney and ligation and excision of the poles of the contralateral kidney. Control rats were subjected to a sham-operation which involved exposure of both kidneys and subsequent closure of the two separate flank incisions. 2°HPT was allowed to develop over the following 6 weeks. Then, the animals which were to be used in the set-point determinations were reanesthetized with the ketamine/xylazine cocktail described above and polyvinyl catheters were implanted chronically in the abdominal aorta (blood sampling) and inferior vena cava (infusions) as described previously (Fox, 1990). The animals were allowed to recover for 3 days prior to the determination of the set-point (Fox, 1991). Briefly, following the collection of a basal blood sample, hypocalcemia (0.8 to 0.9 mM) was induced over 30 to 45 min by the i.v. infusion of EGTA. Then plasma Ca^{2+} levels were increased over 2 to 2.5 h to ~1.5 mM, initially by progressively reducing the EGTA infusion rate and subsequently by infusing Ca gluconate at a progressively increasing rate. Blood samples (0.8 ml) were collected at ~0.1 mM intervals (about every 15 min) as plasma Ca^{2+} levels were raised. Plasma Ca^{2+} levels were measured immediately. Plasma was prepared and stored at −20°C for subsequent PTH assay. For each blood sample, after removal of the plasma sample, the red cell pellet was resuspended in an equal volume of normal rat plasma and returned to the study animals.

At least 3 days after the set-point determination and 7 weeks after the surgical induction of CRI, both catheterized and unstudied rats were anesthetized with the ketamine/xylazine cocktail and killed by exsanguination by cardiac puncture into a heparinized syringe. Plasma Ca^{2+} levels were measured immediately and plasma was stored at −20°C prior to additional analysis. With the aid of a dissecting microscope, the parathyroid glands were separated from associated thyroid tissue, frozen quickly on dry ice, and stored at −90°C until analysis.

Experiment 2. *Regulation of set-point and CaR gene expression by dietary Ca deficiency*

The rats were fed the normal- and low-Ca diets described above for 5 weeks. Then, five animals from each dietary group were catheterized and subjected to a determination of the set-point essentially as described in *Experiment 1*. Most of the rats fed the Ca-deficient diet were already markedly hypocalcemic and did not require an EGTA infusion to initially lower their plasma Ca^{2+} levels. Thus, in contrast to the rats with CRI studied in *Experiment 1* and those fed the normal-Ca semi-synthetic diet, most rats fed the low-Ca diet started this experiment with an i.v. Ca gluconate infusion. However, the majority of the animals in *Experiment 2* did not tolerate the procedure and exhibited signs of distress when plasma Ca^{2+} levels reached ~1.1 to 1.2 mM. They were euthanized before the experiment was completed. The reasons for the problems during the Ca infusions are unclear. Initially, we thought that the Ca infusions may induce severe hypophosphatemia, particularly in the low-Ca group which have a severely demineralized skeleton. However, the rats fed the semi-synthetic normal-Ca diet in which the initial hypocalcemia had to be induced by EGTA infusion reacted similarly and subsequent measurements indicated that plasma phosphate levels actually tended to increase during the Ca infusions (data not shown). Whatever the reasons for the unanticipated adverse reaction, particularly because no difficulties were encountered in chow-fed rats with CRI, and in aged rats studied previously (Fox, 1991), assessment of the set-point in this experiment was impossible. The remaining unstudied rats were anesthetized and blood samples and parathyroid glands were collected for analysis as described in *Experiment 1* above.

Analyses

CaR mRNA levels were quantified using a solution hybridization assay described in detail previously (Rogers *et al.*, 1995). Briefly, individual pairs of parathyroid glands were homogenized and total nucleic acid was extracted with phenol/chloroform and precipitated with ethanol. The RNA was hybridized to a 1.2 kb ^{32}P-labeled riboprobe complementary to -199 to 994 bp of rat kidney CaR (RaKCaR) mRNA (Riccardi *et al.*, 1995). Synthetic full length RaKCaR mRNA was used to generate a standard curve. After hybridization, samples were treated with RNase to digest unhybridized probe. Hybrids were precipitated with trichloroacetic acid, collected on filters, and radioactivity was determined. Parathyroid gland CaR mRNA levels were normalized per μg

DNA to ensure that the measured concentrations would not be influenced by the variable hyperplasia that occurs in the parathyroid glands of rats with 2°HPT.

Plasma Ca^{2+} levels were measured immediately after collection on duplicate 35 μl samples of heparinized whole blood using a Ciba Corning model 634 ionized Ca analyzer. Plasma PTH levels were measured using a rat PTH immunoradiometric assay kit (Immutopics, San Clemente, CA). Plasma PTH levels are expressed as pg-equivalents of rat PTH-(1–34) per ml. Plasma $1,25(OH)_2D_3$ levels were measured using a calf thymus radioreceptor assay kit (Nichols Institute, San Juan Capistrano, CA), BUN levels using a kit (Sigma no. 640-A) and plasma phosphate levels using the method of Chen *et al.* (1956). DNA was determined by fluorometric Hoechst stain assay (Labarca & Paigen, 1980).

All data are expressed as means ± S.E. The significance of differences between groups was determined by *t*-test. Curves were fit to the plasma Ca^{2+} *vs.* PTH data from the set point studies in *Experiment 1* with the four-parameter model described by Brown (1983) using commercial software (KaleidaGraph™; Synergy Software, Reading, PA).

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References

Brown, E.M. (1983). Four parameter model of the sigmoidal relationship between PTH release and extracellular calcium concentration in normal and abnormal parathyroid tissue. *J. Clin. Endocrinol. Metab.*, **56,** 572–581.

Brown, E.M. (1991). Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and the role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiol. Rev.*, **71,** 371–411.

Brown, E.M., Gardner, D.G., Brennan, M.F., Marx, S.J., Spiegel, A.M., Attie, M.F., Downs, R.W., Jr., Doppman, J.L. & Aurbach, G.D. (1979). Calcium-regulated parathyroid hormone release in primary hyperparathyroidism. Studies in vitro with dispersed parathyroid cells. *Am. J. Med.*, **66,** 923–931.

Brown, E.M., Wilson, R.E., Eastman, R.C., Pallotta, J. & Marynick, S.P. (1982). Abnormal regulation of parathyroid hormone release by calcium in secondary hyperparathyroidism due to chronic renal failure. *J. Clin. Endocrinol. Metab.*, **54,** 172–179.

Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M.A., Lytton, J. & Hebert, S.C. (1993). Cloning and characterization of an extracellular Ca^{2+} sensing receptor from bovine parathyroid. *Nature*, **366,** 575–580.

Capuano, I.V., Krapcho, K.J., Hung, B.C., Brown, E.M., Hebert, S.C. & Garrett, J.E. (1994). Characterization of the human calcium receptor gene. *J. Bone Miner. Res.*, **9,** S145 (Abstract).

Chen, P.S., Jr., Toribara, T.Y. & Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.*, **28,** 1756–1758.

Cloutier, M., Gascon-Barré, M. & D'Amour, P. (1992). Chronic adaptation of dog parathyroid function to a low-calcium-high-sodium-vitamin D-deficient diet. *J. Bone Miner. Res.*, **7,** 1021–1028.

D'Amour, P., Weisnagel, J., Rousseau, L., Brossard, J.H., Ste.-Marie, L.-G. & Lepage, R. (1994). The parathyroid function *in vivo* in primary hyperparathyroidism. Comparison with normals. *J. Bone Miner. Res.*, **9,** S337 (Abstract).

Findlay, D.M. & Martin, T.J. (1984). Relationship between internalization and calcitonin-induced receptor loss in T 47D cells. *Endocrinology*, **115,** 78–83.

Fox, J. (1990). Production and metabolic clearance rates of 1,25-dihydroxyvitamin D_3 during maturation in rats: studies using a primed-infusion technique. *Horm. Metab. Res.*, **22,** 278–282.

Fox, J. (1991). Regulation of parathyroid hormone secretion by plasma calcium in aging rats. *Am. J. Physiol.*, **260,** E220–E225.

Fukada, N., Tanaka, H., Tominaga, Y., Fukagawa, M., Kurokawa, K. & Seino, Y. (1993). Decreased 1,25-dihydroxyvitamin D_3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. *J. Clin. Invest.*, **92,** 1436–1443.

Fukagawa, M., Kaname, S., Igarishi, T., Ogata, E. & Kurokawa, K. (1991). Regulation of parathyroid hormone synthesis in chronic renal failure in rats. *Kidney Int.*, **39,** 874–881.

Galper, J.B., Dziekan, L.C., Miura, D.S. & Smith, T.W. (1982). Agonist-induced changes in the modulation of K^+ permeability and beating rate by muscarinic agonists in cultured heart cells. *J. Gen. Physiol.*, **80,** 231–256.

Garrett, J.E., Capuano, I.V., Hammerland, L.G., Hung, B.C.P., Brown, E.M., Hebert, S.C., Nemeth, E.F. & Fuller, F. (1995). Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J. Biol. Chem.*, **270,** 12919–12925.

Goodman, W.G., Belin, T., Ramirez, J.A., Gales, B., Segre, G.V., Jüppner, H. & Salusky, I.B. (1994). A comparison of calcium-regulated PTH release in mild and severe secondary hyperparathyroidism. *J. Am. Soc. Nephrol.*, **5,** 880 (Abstract).

Heath, H., III. (1994). Familial benign hypercalcemia - from clinical description to molecular genetics. *West. J. Med.*, **160,** 554–561.

Heath, H., III., Odelberg, S., Brown, D., Hill, V.M., Robertson, M., Jackson, C.E., Teh, B.T., Hayward, N., Larsson, C., Buist, N., Garrett, J. & Leppert, M. (1994). Sequence analysis of the parathyroid cell calcium receptor (CaR) in familial benign hypercalcemia (FBH): a multiplicity of mutations? *J. Bone Miner. Res.*, **9,** S414 (Abstract).

Khosla, S., Ebeling, P.R., Firek, A.F., Burritt, M.F., Kao, P.C. & Heath, H., III. (1993). Calcium infusion suggests a set-point abnormality of parathyroid gland function in familial benign hypercalcemia and more complex disturbances in primary hyperparathyroidism. *J. Clin. Endocrinol. Metab.*, **76,** 715–720.

Labarca, C. & Paigen, K. (1980). A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.*, **102,** 344–352.

LeBoff, M.S., Shoback, D., Brown, E.M., Thatcher, J., Leombruno, R., Beaudoin, D., Henry, M., Wilson, R., Pallotta, J., Marynick, J., Stock, G. & Leight, G. (1985). Regulation of parathyroid hormone release and cytosolic calcium by extracellular calcium in dispersed and cultured bovine and pathological human parathyroid cells. *J. Clin. Invest.*, **75,** 49–57.

Ledger, G.A., Burritt, M.F., Kao, P.C., O'Fallon, W.M., Riggs, B.L. & Khosla, S. (1994). Abnormalities of parathyroid hormone secretion in elderly women that are reversible by short term therapy with 1,25-dihydroxyvitamin D_3. *J. Clin. Endocrinol. Metab.*, **79,** 211–216.

Marx, S.J., Fraser, D. & Rapoport, A. (1985). Familial hypocalciuric hypercalcemia: mild expression of the gene in heterozygotes and severe expression in homozygotes. *Am. J. Med.*, **62,** 698–706.

Mayer, G.P. & Hurst, J.G. (1978). Sigmoidal relationship between parathyroid hormone secretion rate and plasma calcium concentration in calves. *Endocrinology*, **102,** 1036–1042.

Mithal, A., Kifor, O., Kifor, I., Yassilev, P., Butters, R., Krapcho, K., Simin, R., Fuller, F., Hebert, S.C. & Brown, E.M. (1995). The reduced responsiveness of cultured parathyroid cells to extracellular Ca^{2+} is associated with marked reduction in the expression of extracellular Ca^{2+} sensing receptor messenger ribonucleic acid and protein. *Endocrinology*, **136,** 3087–3092.

Okazaki, T., Igarashi, T. & Kronenberg, H.M. (1988). 5'-Flanking region of the parathyroid hormone gene mediates negative regulation by $1,25\text{-}(OH)_2$ vitamin D_3. *J. Biol. Chem.*, **263,** 2203–2208.

Pearce, S.H.S., Trump, D., Wooding, C., Besser, G.M., Chew, S.L., Heath, D.A., Hughes, I.A. & Thakker, R.V. (1994). Four novel mutations in the calcium-sensing receptor gene associated with familial benign (hypocalciuric) hypercalcaemia. *J. Bone Miner. Res.*, **9,** S145 (Abstract).

Pollak, M.R., Brown, E.M., Chou, Y.-H.W., Hebert, S.C., Marx, S.J., Steinmann, B., Levi, T., Seidman, C.E. & Seidman, J.G. (1993). Mutations in the human Ca^{2+} sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell*, **75,** 1297–1303.

Ramirez, J.A., Goodman, W.G., Gornbein, J., Menezes, C., Moulton, L., Segre, G.V. & Salusky, I.B. (1993). Direct *in vivo* comparison of calcium-regulated parathyroid hormone secretion in normal volunteers and patients with secondary hyperparathyroidism. *J. Clin. Endocrinol. Metab.*, **76,** 1489–1494.

Riccardi, D., Park, J., Lee, W.-S., Gamba, G., Brown, E.M. & Hebert, S.C. (1995). Cloning and functional expression of a rat kidney extracellular calcium polyvalent cation-sensing receptor. *Proc. Natl. Acad. Sci. USA*. **92,** 131–135.

Rogers, K.V., Dunn, C.K., Conklin, R.L., Hadfield, S., Petty, B.A., Brown, E.M., Hebert, S.C. & Fox, J. (1995). Calcium receptor expression in the parathyroid glands and kidney of vitamin D-deficient rats are not regulated by plasma calcium or 1,25-dihydroxyvitamin D_3. *Endocrinology*, **136,** 499–504.

Sanchez, C., Goodman, W.G., Ramirez, J.A., Belin, T., Gales, B., Segre, G.V. & Salusky, I.B. (1994). Dynamic assessment of parathyroid function in dialysis patients with either osteitis fibrosa or adynamic renal osteodystrophy. *J. Bone Miner. Res.*, **9,** S347 (Abstract).

St. John, A., Thomas, M., Dick, I., Young, P. & Prince, R.L. (1994). Parathyroid function in mild to moderate renal failure: evaluation by oral calcium suppression test. *J. Clin. Endocrinol. Metab.*, **78,** 1436–1438.

Teitelbaum, A.P., Silve, C.M., Nyiredy, K.O. & Arnaud, C.D. (1986). Down-regulation of parathyroid hormone (PTH) receptors in cultured bone cells is associated with agonist-specific intracellular processing of PTH-receptor complexes. *Endocrinology*, **118,** 595–602.

Udén, P., Halloran, B., Daly, R., Duh, Q.-Y. & Clark, O. (1992). Set-point for parathyroid hormone release increases with postmaturational aging in the rat. *Endocrinology*, **131,** 2251–2256.

Zhong, M., Finch, J., McCracken, R., Morrissey, J., Slatopolsky, E. & Brown, A.J. (1994). Rat parathyroid gland calcium receptor: regulation by 1,25-dihydroxyvitamin D_3 but not by dietary hyperparathyroidism. *J. Bone Miner. Res.*, **9,** S217 (Abstract).