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Parathyroid gland calcium receptor gene expression is not regulated by increased dietary phosphorus in normal and renal failure rats

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Abstract. The extracellular calcium regulation of parathyroid hormone (PTH) secretion is mediated by a cell surface G-protein-coupled calcium receptor (PCaR). The abnormal calcium sensing in renal failure could be the result of abnormalities of the PCaR. However, PCaR gene expression has been shown to be unaffected by 5/6 nephrectomy in the rat. Whether factors that enhance secondary hyperparathyroidism in renal failure affect the PCaR gene expression is not known. We studied normal rats (sham) ($n=40$) and renal failure rats (3/4 nephrectomy) ($n=40$). Half of the rats in each group received standard (0.6% calcium, 0.6% phosphorus) and half high phosphorus (0.6% calcium, 1.2% phosphorus) diet. Compared to the standard diet, the high phosphorus diet induced secondary hyperparathyroidism both in sham and in renal failure rats (intact PTH: 22.3 ± 2.03 vs 54.3 ± 7.6, $P<0.01$, and 26.2 ± 3.9 vs 178.7 ± 23.2 pg/ml, $P<0.01$, respectively). After the cloning of a rat PCaR cDNA fragment corresponding to the extracellular domain which showed an 89% homology with its bovine counterpart, a probe for Northern blot analysis was obtained. No differences in the PCaR mRNA/18s RNA ratio in high phosphorus compared to the standard diet group were observed, both in sham and in renal failure rats. In conclusion, PCaR gene expression is not involved in the genesis of the secondary hyperparathyroidism induced by a high phosphorus diet. An alternative means of increasing PTH secretion by phosphorus loading could be an alteration of the sensitivity of the PCaR to activation by extracellular calcium.

Key words: parathyroid calcium receptor; renal failure; secondary hyperparathyroidism

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Introduction

The main role of parathyroid hormone (PTH) is to regulate calcium homeostasis, and change in calcium concentration is the primary physiological regulator of PTH secretion. The physiological and biochemical evidence collected over the past decade has led to the view that on the parathyroid cell surface a calcium receptor (CaR) senses the changes in extracellular calcium concentration [1–4]. This has been confirmed by the cloning of a G-protein-coupled CaR from bovine and human parathyroid glands [5,6]. More recently, a rat kidney CaR has been cloned, whose predicted protein shares a 92% homology to bovine parathyroid CaR [7]. Although *in vivo* binding kinetics and the mechanism of signal transduction of parathyroid CaR [PCaR] are still unclear, its crucial role in the maintenance of calcium homeostasis has been evidenced by the demonstration of mutations that either inactivate or overactivate the PCaR in humans [8–10].

Secondary hyperparathyroidism in chronic renal failure is associated with an abnormal ability to sense extracellular calcium concentration [11] and this could be the result of abnormalities in the PCaR. However, secondary hyperparathyroidism induced by 5/6 nephrectomy in the rat did not affect the mRNA of PCaR in one study [12]. Whether factors that enhance secondary hyperparathyroidism in renal failure affect the PCaR gene expression is not known.

Phosphorus retention is an important factor involved in the development of renal hyperparathyroidism and high phosphate diet has been used as the traditional method for the induction of secondary hyperparathyroidism in renal failure [13,14]. Moreover, there is evidence suggesting that hyperphosphataemia can alter the sensitivity of the parathyroid gland to extracellular calcium [15]. Thus we investigated, both in normal rats and in rats with renal failure, whether a decrease of PCaR gene expression is involved in the genesis of secondary hyperparathyroidism induced by a high phosphorus diet. For this purpose we compared steady-

state PCaR mRNA in rats fed with standard or high phosphorus diet. Finally, we provide the cloning of a cDNA fragment of the rat PCaR.

Materials and methods

Animals and diets

Male Wistar rats weighing 200–300 g underwent arterial ligation of the left kidney ($n=40$) or sham operation ($n=40$). This was followed 1 week later by a right nephrectomy or sham operation. The final renal function reduction was 3/4 nephrectomy. During all surgical procedures 50 mg/kg of i.p. sodium pentothal (Abbot Labs, Spain) was used.

Half of the rats in each group received standard diet (0.6% calcium, 0.6% phosphorus), and the other half a high phosphorus diet (0.6% calcium, 1.2% phosphorus) (ICN Biomedicals, CA, USA) for 18 days. Both diets have the same vitamin D content (100 IU/100 mg). This high phosphorus diet adversely affects secondary hyperparathyroidism in rats with renal failure [13,14]. Rats were pair-fed with about 16 g food daily, and received water *ad libitum*. Rats ingesting less than 10 g/day were removed from the study. At the end of the experiment blood was drawn from the aorta under pentothal anaesthesia and the parathyroids were excised with the aid of a dissecting microscope.

Serum determinations

Serum calcium, phosphorus and creatinine were measured by standard autoanalyser procedures. Intact 1-84 rat PTH was determined by an IRMA assay (Nichols Institute, San Juan Capistrano, CA, USA).

Cloning of a rat PCaR cDNA fragment

One cDNA synthesis reaction was carried out using approximately 5 μg total RNA from parathyroid glands following standard procedures [16]. Based on the published sequence of the bovine cDNA PCaR [5] two degenerate oligonucleotide primers were designed spanning 518 bp localized in the extracellular segment, a region expected to conserve its calcium binding ability. Positions of the primers in that sequence were 561 bp for 5′ primer and 1057 bp for 3′ primer from the start codon. In nucleotide positions with high ambiguity, deoxi-inosine was used to allow non-complementary nucleotides to pair [17]. Primer sequences were: PCaR1, 5′ CAT A/T/C CCIAA T/C GA T/C GA A/G CA T/C CA, and PCaR2, 5′ G G/A CA G/A TT G/A AAIGT T/C TC T/C TCCCA.

PCR was carried out in a final volume of 50 μl containing 1 U *Taq* DNA polymerase, 100 μM dNTPs, 1 μM each of the 5′ and 3′ degenerated primers and 1 μl cDNA sample, in a standard buffer (Promega Corp., WI, USA). The amplification was performed on a DNA thermal cycler (Perkin-Elmer/Cetus Corp.), with the following profile: 94°C (1 min), 55°C (1 min), 72°C (1 min), 30 cycles. The PCR product was cloned using TA cloning strategy in pCRII vector (Invitrogen Corp., CA) by means of conventional ligation with T4 DNA ligase and transformation with $CaCl_2$ and heat shock. The cloned insert was sequenced using a Sequenase Kit (Amersham, Germany). The complete sequence of this 518 bp cDNA fragment is presented in Figure 1, along with that reported in bovine.

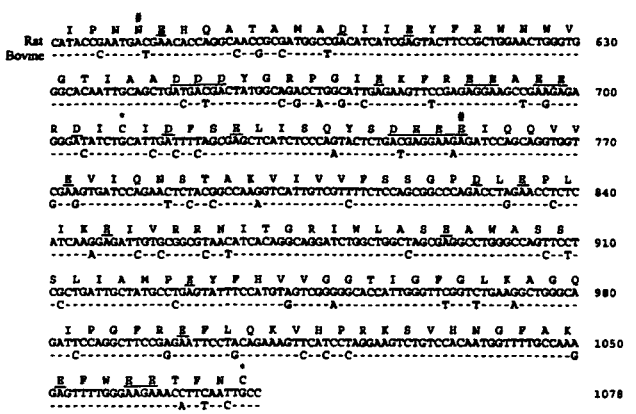

Fig. 1. Nucleotide sequence of the 518 bp rat PCaR cDNA fragment. Nucleotides are numbered coinciding with the bovine PCaR sequence published elsewhere [5] and both sequences are compared. Predicted amino acid sequence (single letter code) for the rat is also shown. Conserved cysteines are labelled (*). Only two amino acids (labelled #) are changed compared to bovine. This fragment contains a highly acidic region, with 28 acidic amino acids (underlined), predicted to be involved in the binding of Ca^{2+} to rat PCaR.

Probe

Two oligonucleotides complementary to sequences flanking the cloning site in pCRII were used. PCR reaction was performed as described above. PCR product was confirmed by polyacrilamide gel electrophoresis and cleaned up with Sephadex G-50 (Pharmacia Biochemicals, WI, USA) by spin column [18]. Probe was labelled by random priming using the Prime-a-Gene kit (Promega Corp.) and 32P-dCTP 3000 Ci/mmol (Amersham). Then the probe was cleaned up by spin column. Specific activity of the probe was typically about 108 cpm/μg.

RNA isolation and Northern analysis

Pools of 10 parathyroid glands were processed. Tissue samples were homogenized and RNA was isolated by extraction with acid-guanidine isothiocyanate-phenol-chloroform [19]. Final RNA pellets were redissolved in 11 μl of Tris–EDTA with 0.5% SDS. The yield of RNA was determined by spectrophotometry [18]. Typically one pool of 10 glands yielded approximately 14–20 μg RNA. Electrophoretic fractionation was performed according to Kroczek and Siebert [20]. About 15 μg total RNA were heat denatured in a buffer containing 63% formamide and 0.02 μg/μl ethidium bromide, loaded onto a submarine 1.2% agarose minigel with 1.1% formaldehyde, and separated by electrophoresis for 2.5 h. Gels were photographed with a 665 type Polaroid film.

The resolved RNA was transferred to Hybond-N membranes (Amersham) by capillary blotting, and covalently cross-linked by UV. The membranes were hydridized and washed as described elsewhere [20]. The membranes were exposed to Cronex X-ray film (Dupont, DE, USA) with intensifying screens during 1 week. To quantitate the mRNA in each blot, X-ray films were subjected to densitometry using a GS-670 Imaging Densitometer (BioRad Labs, CA) aided by the Molecular Analyst software (BioRad Labs). Densitometry of the rRNA 28s from the Polaroid negative film was used to normalize mRNA. All the results were expressed as per cent of controls.

Statistical analysis

The results are presented as $X \pm SE$. Student's unpaired *t*-test was used to compare two group means. A P value <0.05 was considered significant.

Results

Cloning of a 0.5 kb rat PCaR cDNA fragment

We have cloned a 518 bp rat PCaR cDNA fragment corresponding to a 172 amino acid portion of the PCaR protein, localized in the extracellular structural domain. This domain is suspected to be involved in calcium binding. Figure 1 shows the nucleotide and the deduced amino acid sequences of that fragment. An 89% nucleotide and 99% amino acid homology with the bovine sequence was found.

Effects of phosphorus retention on PCaR mRNA

Table 1 shows biochemical data in normal and renal failure rats, fed with standard or high phosphorus diet. PTH concentrations of rats with normal renal function (sham) fed with high phosphorus diet were more than double those of the standard group, while non-significant changes were observed in serum calcium and phosphorus. In renal failure rats, high phosphorus diet induced a significant increase in serum phosphorus and PTH.

Northern blot analysis showed that the PCaR mRNA/RNA 28s ratio was similar in rats fed with standard or high phosphorus diet with normal renal function or renal failure (Figure 2). Moreover, no differences in that ratio was seen between sham and renal failure rats with standard or high phosphorus diet.

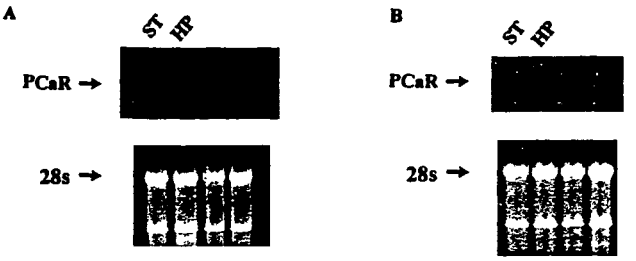

Fig. 2. Northern blot analysis of rat PCaR mRNA. Effect of standard (ST) (lanes 1, 3) and high phosphorus (HP) (lanes 2, 4) diets in sham (A) and renal failure (B) rats. The 28s RNA is shown as a control. In sham animals the mean PCaR mRNA/28s RNA ratio (densitometric analysis) of the HP group was 90% of the SD group. In renal failure the HP group showed a ratio of 99.5% of the SD group.

Discussion

The results of the present study show that secondary hyperparathyroidism induced by a high phosphate diet is not associated with changes in the parathyroid gland CaR mRNA in rats with normal renal function or with renal failure. Thus, changes in the parathyroid CaR gene expression are unlikely to be involved in the genesis of secondary hyperparathyroidism induced by a high phosphate diet. Moreover, we provide the cloning of a 518 bp cDNA fragment of rat parathyroid CaR which shares an 89% homology to its bovine counterpart.

The recent cloning of the CaR from bovine and human parathyroid glands has allowed the study of factors which regulate the expression and function of this receptor. The cloning of a cDNA fragment of the rat parathyroid CaR, corresponding to the extracellular domain, performed in this study provides additional information when a rat model is considered. We have shown that this clone shares an 89% homology with its bovine counterpart, and 99% when amino acid sequence is considered. This level of homology is greater than that found between bovine parathyroid and rat kidney CaR clones.

Modulation of receptor mRNA by physiological regulators of the cells bearing these receptors has been demonstrated for several G-protein-coupled receptors [21,22]. This is one mechanism for modifying receptor protein expression and, hence, the cellular response to agonist. However, in a vitamin D-deficient rat model a wide range of plasma calcium or calcitriol did not regulate the parathyroid gland CaR mRNA [23]. On the other hand, treatment of vitamin D-deficient rats with calcitriol induced a moderate (30–60%) increase in parathyroid CaR mRNA, suggesting that this could be an additional mechanism for controlling PTH secretion [24].

In uraemic hyperparathyroidism there is an abnormal calcium sensing that could be the result of abnor-

Table 1. Biochemical determinations in sham and renal failure rats fed with standard (ST) or high phosphorous (HF) diet

	Normal renal function (sham)		Chronic renal failure	
	ST ($n=20$)	HP ($n=20$)	ST ($n=20$)	HP ($n=20$)
Creatinine (mg/dl)	0.23 ± 0.006	0.22 ± 0.007	0.45 ± 0.01	0.43 ± 0.02
Calcium (mg/dl)	10.02 ± 0.05	9.89 ± 0.07	10.52 ± 0.13	10.26 ± 0.27
Phosphorous (mg/dl)	7.48 ± 0.12	7.94 ± 0.21	7.21 ± 0.20	9.77 ± 0.42*
Intact PTH (pg/dl)	22.30 ± 2.03	54.31 ± 7.6*	26.21 ± 3.9	178.67 ± 23.16*

*$P<0.01$ vs ST.

malities in the CaR. However, parathyroid CaR gene expression has been shown to remain unchanged in rats with 5/6 nephrectomy [12]. In the present study we have confirmed this finding in rats with 3/4 nephrectomy. Thus, alterations in the parathyroid CaR gene expression are unlikely to be involved in the genesis of secondary hyperparathyroidism in this model.

Phosphorus retention is an important factor in the genesis of uraemic secondary hyperparathyroidism. High phosphate diet has been used as the traditional method to enhance secondary hyperparathyroidism in renal failure. Factors involved in the phosphorus loading-induced secondary hyperparathyroidism in renal failure have included a reduction in calcitriol [25], a decreased calcaemic response to PTH [13], and hypocalcaemia [13,14,26]. Moreover, recent studies suggest that phosphorus may directly stimulate PTH secretion as well as gene expression [15,27]. In the present study high phosphate diet induced secondary hyperparathyroidism both in sham and renal failure rats, as has been previously reported [13,14]. Nevertheless, parathyroid CaR mRNA was not modified, suggesting that regulation of parathyroid CaR gene expression is not involved in the genesis of the secondary hyperparathyroidism induced by phosphate loading. An alternative means of increasing PTH secretion by phosphorus loading could be an alteration of the sensitivity of the CaR to activation by extracellular calcium, possibly by protein kinase phosphorylation of the CaR. In fact, in an *in vitro* study, high phosphorus concentration altered the sensitivity of the parathyroid gland to extracellular calcium [15].

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References

1. Brown EM. Extracellular Ca^{2+} sensing, regulation of parathyroid cell function, and role of Ca^{2+} and other ions as extracellular (first) messengers. *Physiol Rev* 1991; 71: 371–411
2. Nemeth EF, Scarpa A. Cytosolic Ca^{++} and the regulation of secretion in parathyroid cells. *FEBS Lett* 1986; 203: 15–19
3. Shoback DM, Membreno LA, McGhee JG. High calcium and others divalent cations increase inositol triphosphate in bovine parathyroid cells. *Endocrinology* 1988; 123: 382–389
4. Brown E, Enyedi P, LeBoff M, Rotberg J, Preston J, Chen C. High extracellular Ca^{++} and Mg^{++} stimulate accumulation of inositol phosphates in bovine parathyroid cells. *FEBS Lett* 1987; 218: 113–118
5. Brown EM, Gamba G, Riccardi D, Lonbardi M, Butters R, Kifor O, Sun A, Hediger MA, Lytton J, Hebert SC. Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature* 1993; 366: 575–580
6. Garret JE, Hung BC, Brown EM, Hebert CS, Fuller F, Nemeth EF. Cloning and expression of a G-protein-coupled calcium receptor from a human parathyroid adenoma [Abstract]. *J Bone Miner Res* 1993; 8: S148
7. Riccardi D, Park J, Lee WS, Gamba G, Bown EM, Hebert SC. Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proc Natl Acad Sci USA* 1995; 92: 131–135
8. Pollak M, Brown EM, Chou YW, Hebert SC, Marx S, Steinmann B, Levi T, Seidman CE, Seidman JG. Mutations in the human Ca^{2+}-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism. *Cell* 1993; 75: 1297–1303
9. Pollak MR, Brown EM, Estep HL, McLaine PN, Kifor O, Park J, Hebert SC, Seidman CE, Seidman JG. Autosomal dominant hypocalcemia caused by a Ca^{2+}-sensing receptor gene mutation. *Nature Genet* 1994; 8: 303–307
10. Brown EM, Pollak M, Seidman CE, Seidman JG, Chou YW, Riccardi D, Hebert SC. Calcium-ion-sensing cell surface receptors. *N Engl J Med* 1995; 333: 234–240
11. Felsenfeld AJ, Llach F. Parathyroid gland function in chronic renal failure. *Kidney Int.* 1993; 43: 771–789
12. Fox J, Brown EM, Hebert SC, Rogers KV. Parathyroid gland calcium receptor gene expression is unaffected by chronic renal failure or low dietary calcium in rats [Abstract]. *J Am Soc Nephrol* 1994; 5: 879
13. Rodriguez M, Martin-Malo A, Martinez ME, Torres A, Felsenfeld AJ, Llach F. Calcemic response to parathyroid hormone in renal failure: Role of phosphorus and its effect on calcitriol. *Kidney Int* 1991; 40: 1055–1062
14. Bover J, Rodriguez M, Trinidad P, Jara A, Martinez ME, Machado L, Llach F, Felsenfeld AJ. Factors involved in the development of secondary hyperparathyroidism during graded renal failure in the rat. *Kidney Int* 1994; 45: 953–961
15. Almaden Y, Canalejo A, Hernandez A, Garcia MS, Torres A, Rodriguez M. Direct effect of phosphorus on parathyroid hormone secretion of rat parathyroid glands *in vitro*. *J Bone Miner Res*, submitted
16. Krug MS, Berger SL. First-strand cDNA synthesis primed with oligo(dT). *Meth Enzymol* 1987; 152: 316–325
17. Ohtsuka E, Matsuki S, Ikehara M, Yoosuke T, Matsubara K. An alternative approach to deoxyoligonucletides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. *J Biol Chem* 1985; 260: 2605–2608
18. Maniatis T, Fritsch PH, Sambrook J (eds). *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY, 1982
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156–159
20. Kroczek RA, Siebert E. Optimization of northern analysis by vacuum-blotting, RNA-transfer visualization, and ultraviolet fixation. *Anal Biochem* 1990; 184: 90–95
21. Habecker BA, Nathanson MN. Regulation of muscarinic acethylcholine receptor mRNA expression by activation of homologous and heterologous receptors. *Proc Natl Acad Sci USA* 1992; 89: 5035–5038
22. Yang J, Tashjian AH. Regulation of endogenous thyrotropin-releasing hormone (TRH) receptor messenger RNA by TRH in GH4C1 cells. *Mol Endocrinol* 1993; 7: 753–758
23. Rogers KV, Dunn CK, Conklin RL, Hadfield S, Petty BA, Brown EM, Hebert SC, Nemeth EF, Fox J. Calcium receptor messenger robonucleic acid levels in the parathyroid glands and kidney of vitamin D deficient rats are not regulated by plasma calcium or 1,25-dihydroxyvitamin D3. *Endocrinology* 1995; 136: 499–504
24. Zhong M, Finch J, McCracken R, Morrisey J, Slatopolsky E, Brown A. The calcium receptor of rat parathyroid glands is regulated by 1,25-Dihydroxy-Vitamin D3 but not by dietary hyperparathyroidism [Abstract]. *J Am Soc Nephrol* 1994; 5: 892
25. Portale AA, Boothe BE, Halloran BP, Morris RC Jr. Effect of dietary phosphorus on circulating concentrations of 1,25-dihydroxy-vitamin D and immunoreactive parathyroid hormone in children with moderate renal insufficiency. *J Clin Invest* 1984; 73: 1580–1589
26. Slatopolsky E, Calgar S, Pennel JP, Tagart DD, Canterbury JM, Reiss E, Bricker NS. On the pathogenesis of hyperparathyroidism in chronic renal insufficiency in the dog. *J Clin Invest* 1971; 50: 492–499
27. Kilav R, Silver J, Naveh-Many T. Parathyroid hormone gene expression in hypophosphatemic rats. *J Clin Invest* 1995; 96: 327–333