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

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 (Abbott 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, deoxy-inosine was used to allow non-complementary nucleotides to pair [17]. Primer sequences were: PCaR1, 5' CAT A/T/C CCAAA T/C GA T/C GA A/G CA T/C CA, and PCaR2, 5' G G/A CA G/A TT G/A AAAGT T/C TC T/C TCCCA.

PCR was carried out in a final volume of 50 μl containing 1 U Tag 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 (PerkinElmer/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 CaCl2 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.

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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 (#) 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 Ca2+ 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-Gen 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 hybridized 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.

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

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<tr>
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<th>Normal renal function (sham)</th>
<th>Chronic renal failure</th>
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<tr>
<td></td>
<td>ST ($n=20$)</td>
<td>HP ($n=20$)</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>0.23 ± 0.006</td>
<td>0.22 ± 0.007</td>
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<tr>
<td>Calcium (mg/dl)</td>
<td>10.02 ± 0.05</td>
<td>9.89 ± 0.07</td>
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<tr>
<td>Phosphorous (mg/dl)</td>
<td>7.48 ± 0.12</td>
<td>7.94 ± 0.21</td>
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<td>Intact PTH (pg/dl)</td>
<td>23.30 ± 2.03</td>
<td>54.31 ± 7.6*</td>
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<td>0.45 ± 0.01</td>
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<td>10.52 ± 0.13</td>
<td>10.26 ± 0.27</td>
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<td>7.21 ± 0.20</td>
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<td>2621 ± 3.9</td>
<td>178.67 ± 23.16*</td>
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*P<0.01 vs ST.
nalities 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 induced 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 calcemic 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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