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RAPID COMMUNICATION

Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism

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Depressed expression of calcium receptor in parathyroid gland tissue of patients with hyperparathyroidism. The factors involved in abnormal parathyroid cell secretory function and growth in patients with primary (I°) and secondary (II°) hyperparathyroidism are still incompletely understood. We compared the expression of the calcium-sensing receptor (CaR) at the gene message and the protein level in parathyroid tissue obtained from patients with I° non-uremic or II° uremic hyperparathyroidism with that in normal parathyroid tissue, using *in situ* hybridization and immunohistochemistry techniques. The expression of the CaR mRNA and protein was reduced in most cases of I° adenoma and II° hyperplasia, compared with strong expression in normal parathyroid tissue. In II° hyperparathyroidism, expression of both receptor mRNA message and protein was often particularly depressed in nodular areas, compared with adjacent non-nodular hyperplasia. Decreased Ca-R expression in adenomatous and hyperplastic parathyroid glands would be compatible with a less efficient control of PTH synthesis and secretion by plasma calcium than in normal parathyroid tissue.

Our understanding at the cellular and molecular levels of the receptor-mediated effects of calcium and calcitriol on the pre-proparathyroid hormone gene and the gene product, parathyroid hormone (PTH), has substantially improved during recent years [1]. However, the escape from physiologic control of the secretory function and growth of parathyroid tissue in hyperparathyroid disease states is still incompletely understood.

In secondary (II°), reactive forms of hyperparathyroidism, low plasma levels of calcium and/or calcitriol clearly play a major role in stimulating PTH synthesis and secretion, and probably also in enhancing parathyroid hyperplasia [2]. An abnormally low expression of the vitamin D receptor (VDR) has been proposed as another possible mechanism, which could be limited to areas of nodular transformation [3]. VDR expression in primary (I°) hyperparathyroidism has been examined in one study [4], but no comparison was made with normal parathyroid tissue. Whether changes in the expression of the calcium-sensing receptor (CaR), which recently has been cloned by Brown et al [5], occur in I° or II° hyperparathyroidism is unknown at present. Interestingly, the expression of another calcium sensing protein (CAS), which belongs to the low-density-lipoprotein receptor superfamily [6], has been found to be diminished in I° hyperparathyroidism [7].

The purpose of the present study was to examine the expression of the CaR gene message and protein in parathyroid tissue of patients with I° parathyroid adenoma and normal renal function and in that of uremic patients with II° hyperparathyroidism, compared with expression in normal human parathyroid tissue. The recognition of an abnormal CaR expression could help to obtain a better insight into the mechanisms involved in abnormal parathyroid secretory function and growth.

Methods

Patients

Normal parathyroid glands were obtained from 8 patients during surgery for various thyroid diseases. Pathologic parathyroid gland tissue was sampled in 16 patients with I° hyperparathyroidism and normal renal function (all single adenomas) and in 13 patients with uremic II° hyperparathyroidism (diffuse hyperplasia with or without nodule formations). Mean ± SEM ages were 58 ± 4 and 45 ± 6 years in patients with I° and II° hyperparathyroidism, respectively. Mean plasma total calcium levels were 2.87 ± 0.09 and 2.55 ± 0.06 mM, and mean plasma phosphate levels 0.79 ± 0.05 and 2.47 ± 1.5 mM, respectively. Mean plasma immunoreactive PTH1-84 levels were elevated in all cases: 117 ± 10 and 939 ± 120 pg/ml, respectively (normal range 10 to 65 pg/ml). Uremic patients underwent intermittent hemodialysis treatment for variable time periods (mean 5.2 ± 1.2 years).

Tissue fragments were frozen after surgical removal and stored at −80°C. Histopathologic examination by light microscopy was routinely performed on each tissue sample using standard techniques.

Oligonucleotide probes

Two calcium receptor (CaR) antisense 22-mer oligonucleotides corresponding respectively to bases 2412 to 2433 (oligo 1; 5′-TTGCGGAACTTGATGAAGACGC-3′) and to bases 2928 to 2949 (oligo 2; 5′-ATGCTGAAGGTGATGAACTTGG-3′) were synthesized. The sequences were obtained from Gene Data Bank, code gb/S67307 (NCBI, Bethesda, MD, USA). Terminal transferase was used to label probes with digoxigenin-11-dUTP and dATP following manufacturer's recommendations (Boehringer, Mannheim, Germany).

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In situ *hybridization*

Hybridization was performed using frozen sections from 4 patients with normal parathyroids, 6 patients with I° hyperparathyroidism and 4 patients with II° hyperparathyroidism. *In situ* hybridization was performed according to a previously described method [8], with minor modifications. Briefly, 5 μ-thick cryostat sections were fixed in 4% paraformaldehyde-PBS for 10 minutes, rinsed in PBS and prehybridized during one hour at room temperature in a mixture containing 4 × SSC and 1% Denhard's solution. Adjacent sections of each block were mounted on five different slides. Two of them were treated with specific oligonucleotide probes in two different assays, and the other three were control slides.

Hybridization was carried out at 37°C for 16 hours in 50% formamide, 1% Denhardt's solution, 15% SSC, 10 mg/ml yeast transfer RNA, 10 mg/ml salmon sperm DNA and 4% Sarkosyl. Each section was covered with 100 μl of the hybridization solution containing 150 pmol/ml of the appropriate DIG-labeled antisense oligonucleotide probe.

After hybridization, sections were washed two times in 2 × SSC at room temperature, followed by two washes in 1 × SSC, one wash in 0.5 × SSC and a final wash in 0.1 × SSC at 37°C during 30 minutes.

Sections were then equilibrated in buffer A (150 mM NaCl, 100 mM Tris-HCl), supplemented with 2% of normal sheep serum and 0.3% of Triton X-100, at room temperature during one hour and incubated overnight at 4°C with 1% of normal sheep serum, 0.3% Triton X-100 and anti-DIG Fab-fragment–alkaline phosphatase conjugate. After several washes the sections were incubated in darkness during two hours in solution containing NBT (nitro blue tetrazolium) and 5-bromo-4 chloro-3-indolyl phosphate (BCIP) substrate. After developement, sections were washed in buffer and mounted in Aquatex. Controls of the specificity of *in situ* hybridization findings were obtained as follows: (1) no signal after preincubation in a solution containing 150 pg/ml of labeled probe and 50-fold concentrated unlabeled oligonucleotide; (2) no signal when omitting oligonucleotide probe; and (3) no signal after RNAse pre-treatement of tissue sections.

Anti-CaR antibody

A polyclonal anti-CaR specific antibody was used that was generated in rabbits with a fusion peptide (a GST fusion expressed in *Escherichia coli*) representing amino acids 340 to 620 of the human CaR [9]. (The antiserum was kindly provided by Edward Nemeth, M.D., NPS Pharmaceuticals, Salt Lake City, USA).

Immunohistochemistry

For immunohistochemical staining a series of parathyroid tissue fragments from 8 patients with normal parathyroids, 10 patients with I° hyperparathyroidism and 11 patients with II° hyperparathyroidism were sectioned at 5 μm in a cryostat and treated with 0.3% H_2O_2 in methanol for 20 minutes. After incubation in blocking solution, the slides were exposed to the first antibody, directed against the CaR, for 40 minutes in a humidified chamber at room temperature. They were then rinsed and incubated with a second antibody (peroxidase-conjugated goat antirabbit IgG) for 30 minutes. Immunoreactivity was revealed using AEC kit solution (Vector, Burlingame, VT, USA) The specificity of the antibody has been assessed previously [9]. In addition, it was ascertained in the present study by negative controls using preimmune rabbit serum.

Table 1. Ca-sensing receptor mRNA expression, as evaluated by *in situ* hybridization (ISH), in parathyroid tissue samples from patients with normal parathyroid glands (normal), primary (I°) hyperparathyroidism, and secondary (II°) hyperparathyroidism

			II° hyperparathyroidism (N = 4)	
Parathyroid Tissue	Normal (N = 4)	I° hyperparathyroidism (N = 6)	Diffuse hyperplastic area	Nodular hyperplastic area
Number of ISH signals	14 ± 3	5 ± 1	11 ± 3	7 ± 3
Mean optical density	52 ± 4.9	15 ± 0.7	22 ± 8.4	21.5 ± 7.7

Data are mean ± SEM.

Quantification of in situ *hybridization and immunohistochemical signals*

Staining intensity of parathyroid tissue sections after treatment by *in situ* hybridization and immunohistochemistry, respectively, was evaluated by three independent observers. Three to four sections per specimen were assayed. For immunohistochemistry, the presence of perinuclear ring-shaped staining was evaluated for each parathyroid cell, at a magnification ×400, and the ratio of positive to negative cells was established. A total number of approximately 400 cells was counted for each tissue section. For *in situ* hybridization, the number of colorimetric signals forming patches per cell was counted on each tissue section, at a magnification of ×1000. Hybridization signal numbers were then averaged, and mean values calculated. In parallel, tissue sample staining density was determined by a computer-based image analysis software developed by one of us according to Mize, Holdefer and Nabors [10] with minor modifications. Images were grabbed using a Leica DMR microscope Leica (Leica, Germany) and a monochrome Photometrics CCD camera (Photometrics, USA). For each specimen, a minimum of three fields was measured. The mean optical density value for negative controls was 8.2 ± 6.7. Checking of potential errors in image analysis was performed by comparison of repeat measurements of the same slide at different days, yielding a variation coefficient of 7%.

Statistical evaluation of results obtained was done using Student's unpaired or paired t-test, as appropriate.

Results

Light microscopy analysis

In all cases of hyperparathyroidism studied, the diagnosis of predominant chief cell adenoma or hyperplasia was confirmed by histologic examination of paraffin-embedded tissue sections after hematoxylin-eosin staining. The cellular arrangement showed a variety of aspects including nodular, acinar, follicular and trabecular patterns. Areas of oxyphil cells were detected in approximately one third of the adenomas studied. Nodular formations

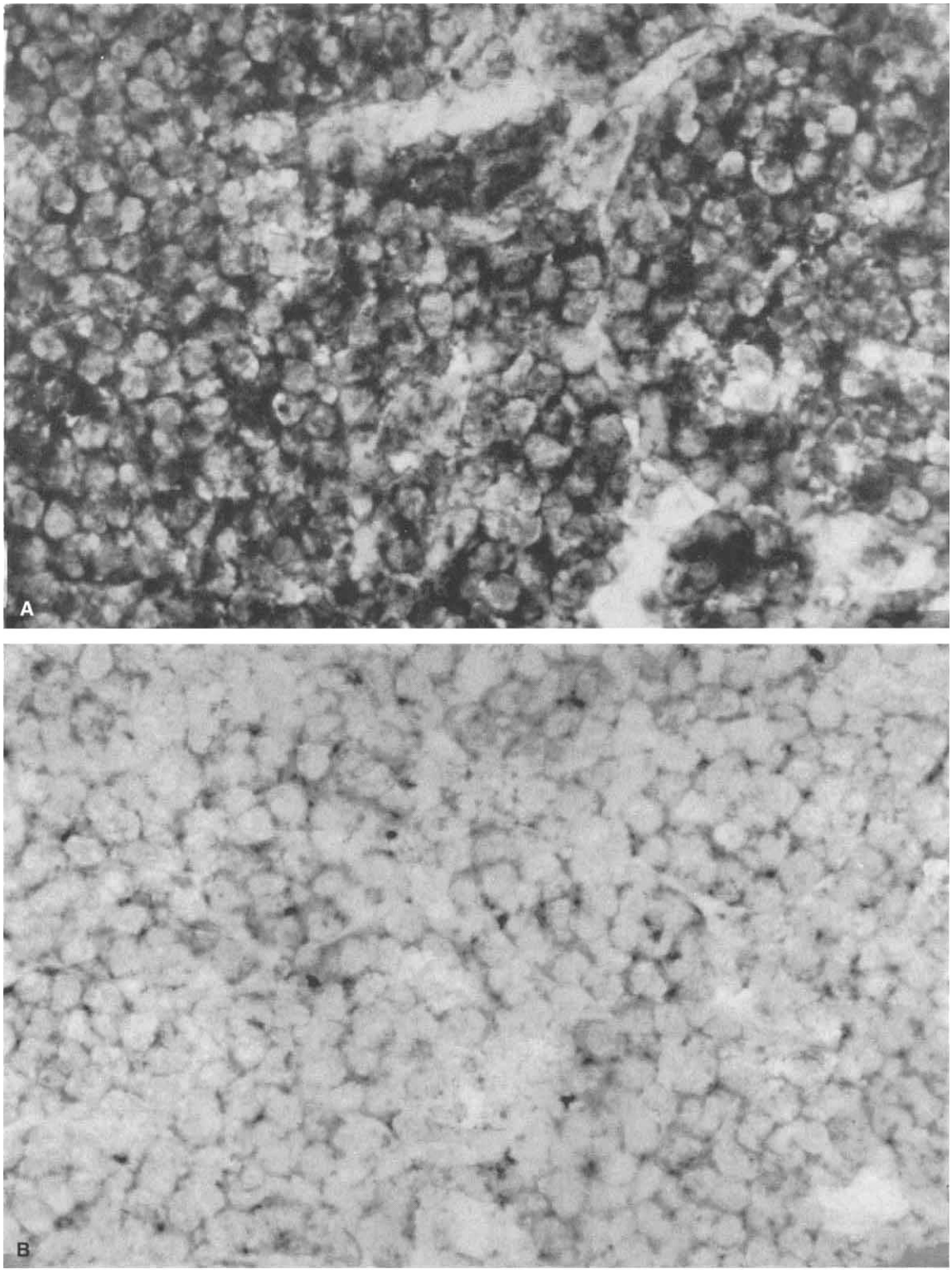

Fig. 1. *Characteristic feature of CaR mRNA expression by in situ hybridization in parathyroid tissue.* (**A**) Normal parathyroid gland, (**B**) primary hyperparathyroidism (solitary adenoma), and (**C**) secondary hyperparathyroidism (diffuse hyperplasia with nodule formation, uremic patient). The strong staining pattern in (A) contrasts with extremely weak staining in (B) and moderate staining in (C). Note the relative decrease of CaR signal in (C) in nodular area (right side), compared with that in adjacent, diffusely hyperplastic area (left side) and absence of signal over fibrous tissue. The photomicrographs were taken at a magnification of ×400.

mainly composed of chief cells were also observed in five of the hyperplastic glands from uremic patients with II° hyperparathyroidism.

Expression of CaR gene message

The hybridization signals, usually forming blue purple patches, were mostly confined to the cytoplasm of the chief cells, while

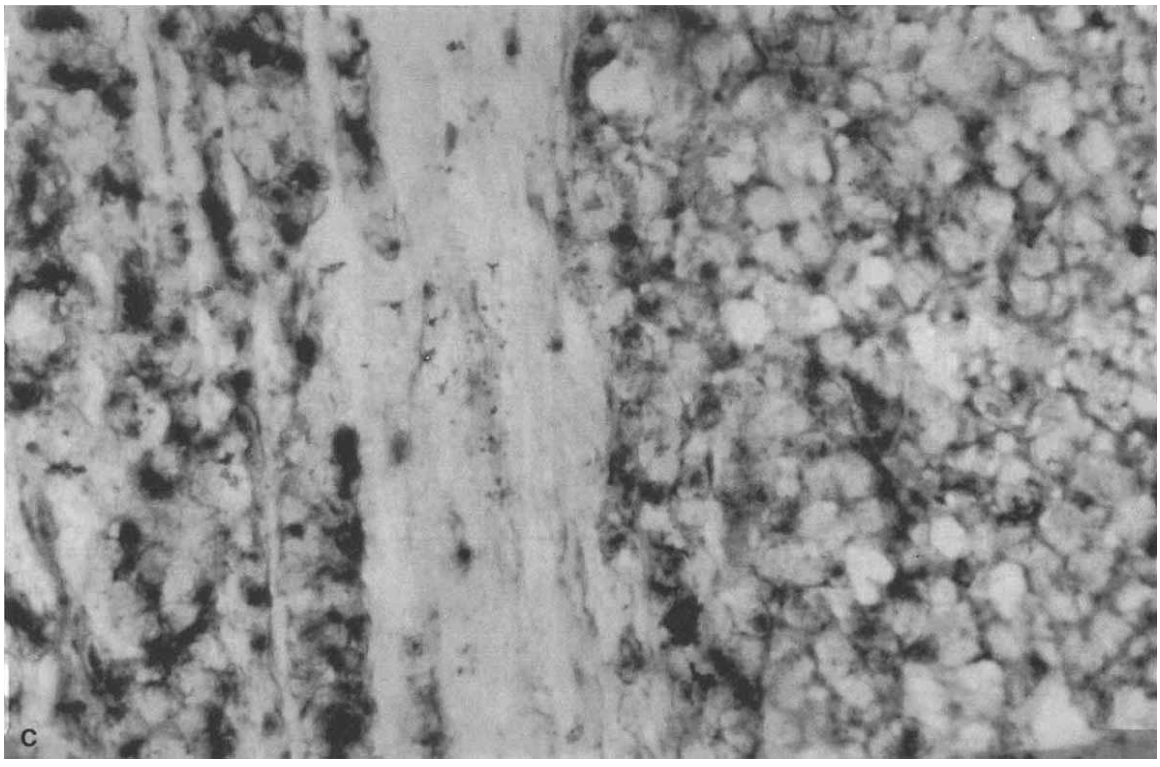

Fig. 1. *Continued.*

mesenchymal elements were not labeled. A strong expression of CaR mRNA by *in situ* hybridization was observed in the 4 samples of normal parathyroid tissue. In contrast, CaR mRNA expression was markedly decreased in the 6 samples of adenomatous tissue of patients with I° hyperparathyroidism. Expression was also decreased in the 4 samples of parathyroid tissue of uremic patients with severe II° hyperparathyroidism. Overall differences in mRNA expression are shown in Table 1, as evaluated by either counting numbers of hybridization signals or by computer-assisted image analysis. In some instances, the decrease was more pronounced in nodular areas than in surrounding areas of diffuse hyperplasia using light microscopy. However, such a difference was not found using image analysis. Figure 1 shows a typical pattern for each patient category, with a nodular and diffuse hyperplastic area in the tissue from a patient with II° hyperparathyroidism.

Expression of CaR protein

CaR protein immunostaining was mainly limited to the area of membrane and cytoplasm in parathyroid cells of all tissue samples examined. A strong CaR staining was detected in the 8 samples of normal parathyroid tissue, whereas staining intensity was markedly diminished in most cases of I° and II° hyperparathyroidism. Quantative differences in CaR protein staining are shown in Figure 2. For II° hyperparathyroidism samples, tissue areas were analyzed as a whole, including diffuse hyperplasia and nodular structures. However, since staining intensity often was lower at weak magnification in well-circumscribed nodular areas in only 5 tissue samples, we then analyzed those areas separately from diffusely hyperplastic areas. The nodular formations in hyperplastic glands from II° hyperparathyroidism patients exhibited a particularly weak staining of CaR, as shown in Figure 3. Cells composing vessel walls, perivascular stroma and capsular fibrous tissue stained negative for the CaR protein. Figure 4 shows a characteristic feature for each type of tissue.

Discussion

The present study demonstrates a decrease of parathyroid CaR mRNA expression, based on an *in situ* hybridization technique, in both I° and II° hyperparathyroidism as compared to normal parathyroid tissue. Our study further shows that the decrease is not limited to the gene message, but that it is present, in both types of disease, at the receptor protein level as well. The decrease of CaR protein expression was particularly pronounced in nodular areas of hyperplastic glands from uremic patients with II° hyperparathyroidism.

Mutations of the CaR gene are associated with abnormal PTH secretion, and different mutations may be associated either with hyperparathyroidism [11] or hypoparathyroidism [12]. The full knock-out of the CaR gene in a mouse model was shown to induce a picture comparable to that of severe neonatal hyperparathyroidism [13]. Recently, loss of heterozygosity for genetic markers flanking the CaR gene locus has been found in a small number of patients with I° hyperparathyroidism in one series [14], whereas in another series no evidence was found for somatic mutations of the CaR gene [15]. However, several gene transcripts of different sizes have been observed in a parathyroid adenoma, in addition to the main transcript of 5.4 kilobases [16].

Our demonstration of a diminished expression of the CaR gene message and its protein product in hyperplastic parathyroid tissue

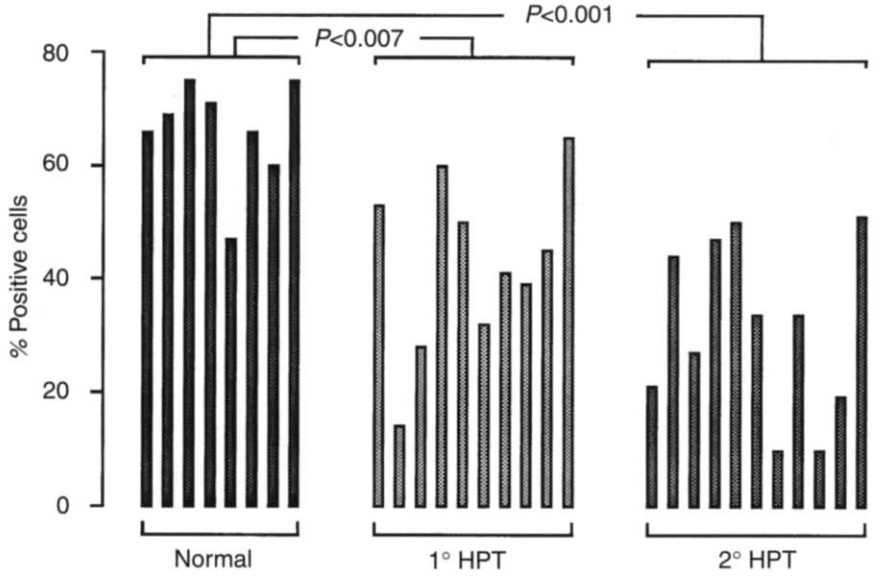

Fig. 2. *Histogram of CaR protein expression by immunohistochemistry, expressed as percent positive cells of total number of cells examined, in parathyroid tissue from patients with normal parathyroid glands, I° hyperparathyroidism (solitary adenoma), and II° hyperparathyroidism.*

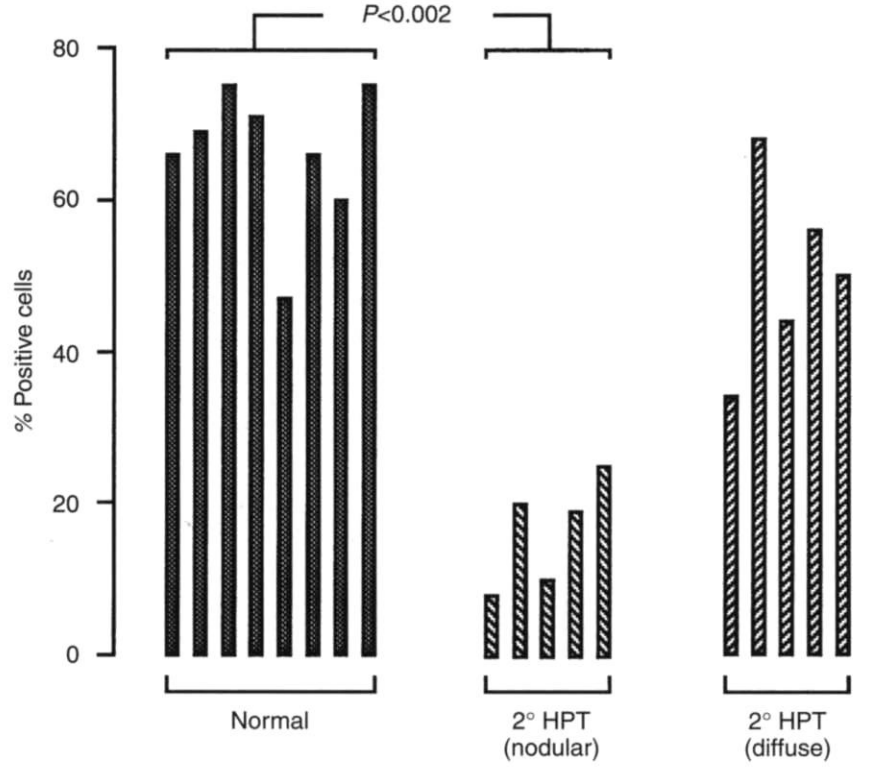

Fig. 3. *Histogram of CaR protein expression by immunohistochemistry, expressed as percent positive cells of total number of cells examined, in parathyroid tissue from patients with normal parathyroid glands and from patients with II° hyperparathyroidism in whom two subsets of parathyroid glands have been analyzed separately, namely diffuse hyperplasia with nodule formation and diffuse hyperplasia without nodule formation.*

could explain observations of an abnormal sensing of the extracellular calcium concentration. A previous *in vitro* study showed a shift in the relationship between ionized calcium and PTH secretion in parathyroid glands sampled from patients with I° or II° hyperparathyroidism [17]. Similarly, abnormal calcium sensing by the parathyroids has been described in such patients *in vivo* [18, 19], even though not all authors would agree, at least for uremic patients with severe II° hyperparathyroidism [20]. Patient-to-patient heterogeneity and differences in disease severity may explain these apparently discrepant observations [21, 22].

The present finding of a decreased CaR expression in chronic dialysis patients is in apparent contradiction with a recent report of no change of CaR expression in rats with chronic renal failure [23]. Differences in the degree of renal failure and the duration and degree of II° hyperparathyroidism might account for this discrepancy.

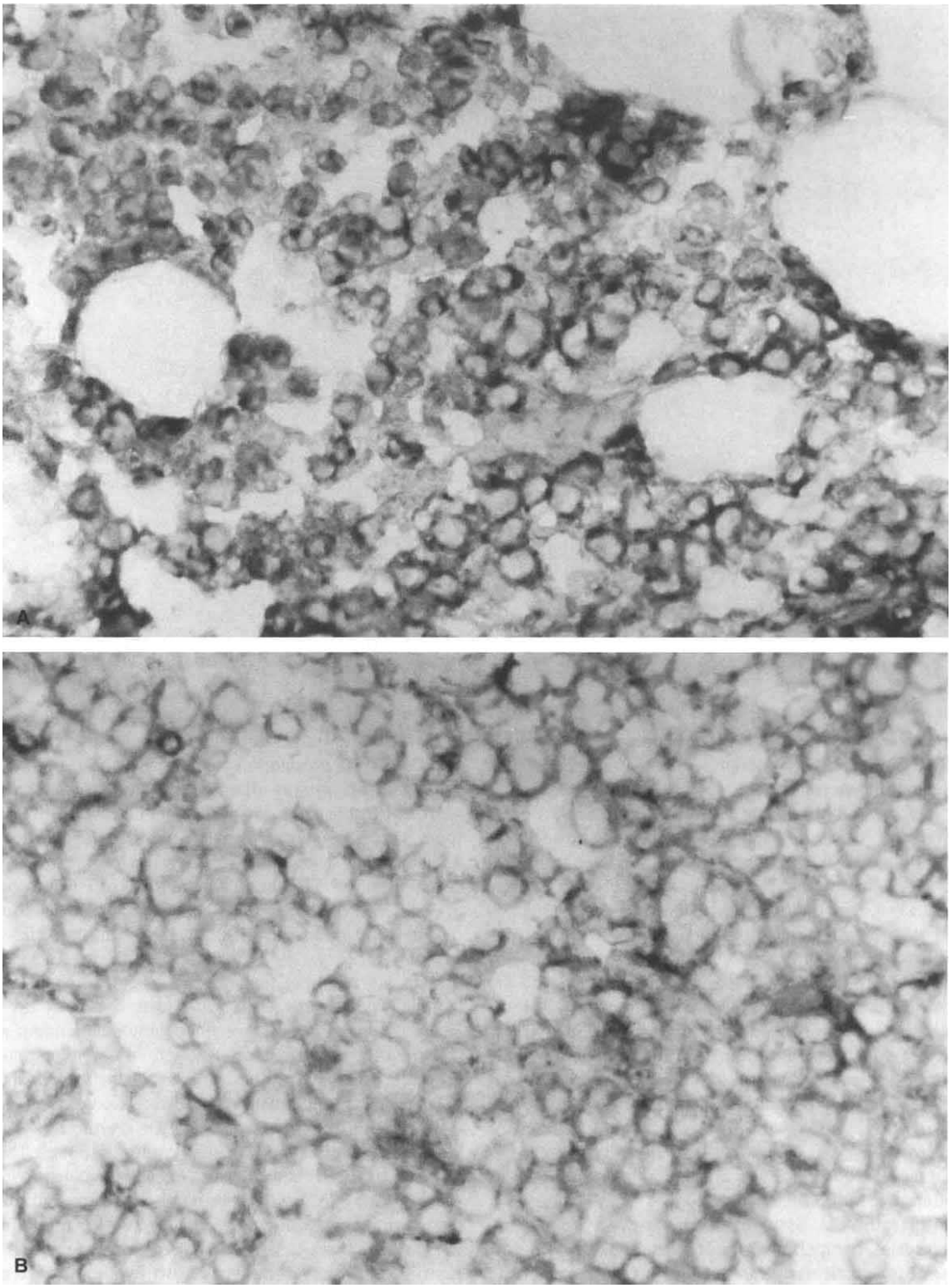

Fig. 4. *CaR protein expression by immunohistochemistry in parathyroid tissue from* (**A**) *normal parathyroid gland,* (**B**) *I° hyperparathyroidism, and* (**C**) *II° uremic hyperparathyroidism (diffuse hyperplasia).* The strong staining pattern in (A) contrasts with weaker staining in (B) and extremely weak staining in (C). The photomicrographs were taken at a magnification of ×400.

The staining intensity of the CaR protein in normal parathyroid tissue was strong and the cellular staining pattern was ring-shaped, possibly corresponding to predominant receptor location in the cell membrane. In contrast, the staining intensity was generally weak in pathologic tissue from patients with I° and II° hyperparathyroidism and the expression pattern was more diffuse

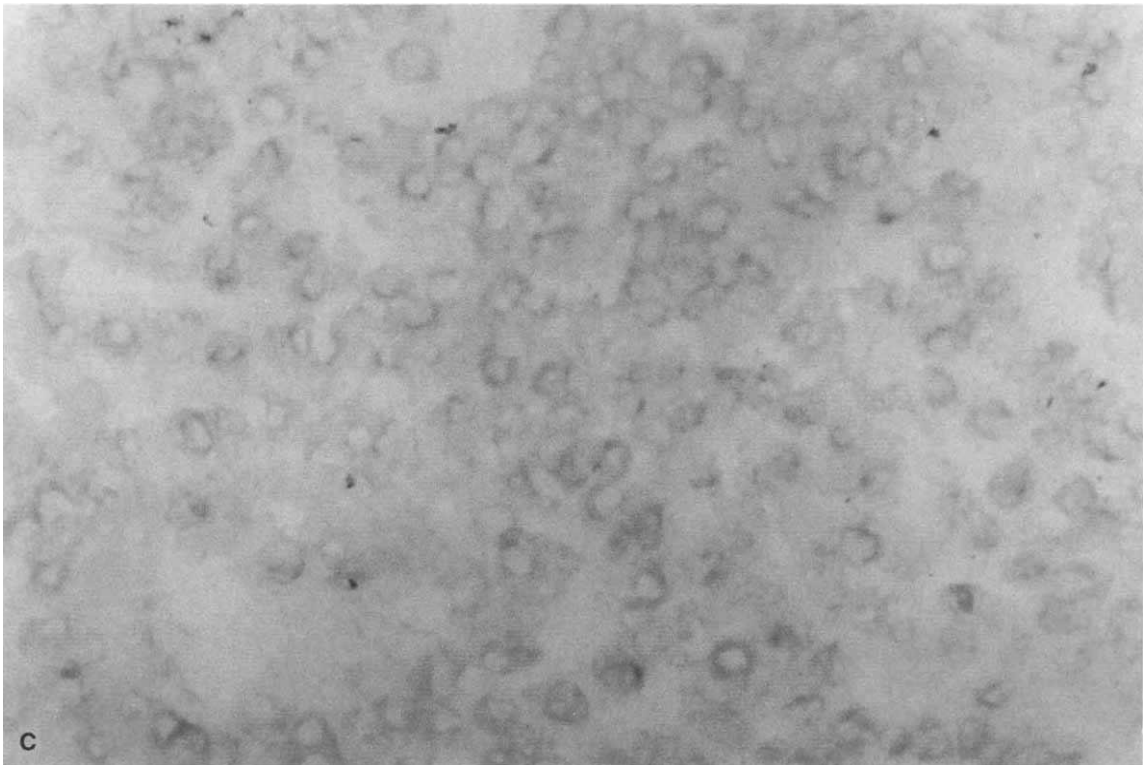

Fig. 4. *Continued.*

accross the cytoplasm, instead of a predilection for the cell membrane. The reason for the decrease in the CaR mRNA and protein expression in hyperplastic parathyroid tissue is not clear at present. The factors regulating CaR expression have not yet been identified. Whereas calcium does not appear to be directly involved [23] a potentially regulatory role of calcitriol is presently under debate [23, 24]. It is also possible that modulation of CaR biosynthesis occurs through alternative RNA processing [16].

A down-regulation of the receptor for the other major modulator of parathyroid function, namely calcitriol, was first shown several years ago in the parathyroid tissue of patients with II° hyperparathyroidism, using a vitamin D binding technique, compared with its expression in I° hyperparathyroidism [4]. However, no normal parathyroid tissue was examined for comparison. This finding has been subsequently questioned [25]. More recently, decreased VDR protein expression has been shown to occur in nodular areas of parathyroid glands in uremic patients with severe II° hyperparathyroidism, but not in diffusely hyperplastic areas [3]. Again, normal parathyroid tissue was not available. In preliminary studies, we found a marked down-regulation of VDR mRNA expression in parathyroid tissue of patients with I° and II° hyperparathyroidism, compared with normal tissue [26].

The findings of a particularly marked down-regulation of the CaR and the VDR in nodular areas of parathyroid tissue from patients with II° hyperparathyroidism would be compatible with an abnormal regulation of receptor expression, perhaps associated with an autonomous growth pattern, although in our experience no good correlation existed between the clonal type of parathyroid tissue growth and the histologic finding of a nodular or diffusely hyperplastic growth [27].

To date, the question remains largely unsolved of whether the excessive PTH production characteristic of various hyperparathyroid states is mainly due to an unopposed increase of PTH synthesis and/or secretion at the cellular level, or if it is mainly due to an increase in parathyroid cell mass, in the absence of excessive PTH synthesis per cell [28]. The former hypothesis would be compatible with a pathologic regulation of PTH production at the cellular level by factors normally involved in its control such as calcium and calcitriol. The latter hypothesis could imply a disturbed local or systemic regulation by other factors involved in cell growth and viability, in addition to calcium and calcitriol. Recently, Naveh-Many et al [29] have shown in rat studies that calcium and phosphate are important independent actors in the control of parathyroid cell growth. Whereas a phosphate-poor diet depressed cell proliferation, a diet rich in phosphate or poor in calcium stimulated it markedly. This was true for rats with normal renal function as well as for uremic rats. A decrease in CaR expression would be able to potentiate the effect of calcium deficiency. Other factors are potentially involved in enhanced cell proliferation. We have observed an increased expression of transforming growth factor-α (TGF-α) mRNA and protein in the parathyroid tissue of uremic patients with severe II° hyperparathyroidism [30]. Obviously, the two hypotheses are not mutually exclusive.

The excessive PTH secretion of primary (I°) parathyroid adenoma has been shown to be associated with uncontrolled parathyroid cell growth which is monoclonal in nature [31]. The *PRAD1/cyclin D1* oncogene, which is abnormally placed under the influence of the regulatory region of the PTH gene, could be responsible in at least a subgroup of patients with I° adenoma [32].

More unexpectedly, sporadic, diffuse I° parathyroid hyperplasia and even the II° parathyroid hyperplasia of chronic renal failure recently have been shown by us to often exhibit a monoclonal growth pattern [27]. Possible underlying somatic mutations of specific suppressor or enhancer genes await elucidation. Whether underlying mutations or deletions of the CaR gene or changes in RNA processing are involved remains to be seen. Mutations of the gene, if any, do not appear to be frequently involved [33]. To our knowledge, the possibility of a somatic mutation of the VDR gene has not yet been addressed. Interestingly, a greater prevalence of the VDR genotype bb recently has been found in postmenopausal women with I° hyperparathyroidism than in appropriate female control subjects [34].

Since the submission of our article, Kifor et al [35] have published a paper reporting a decrease in CaR protein expression in parathyroid tissue from patients with I° as well as with II° hyperparathyroidism, identical to our finding. However, they used biopsies of non-adenomatous, probably functionally less-active parathyroid glands obtained from their patients with I° hyperparathyroidism, whereas we used normal parathyroid glands obtained from patients operated upon for thyroid disease. In addition, the present study provides evidence that the decrease of CaR expression also occurs at the gene message level in both I° and II° hyperparathyroidism.

In conclusion, the present finding of a decreased expression of the CaR gene message and protein in the hyperplastic parathyroid tissue of non-uremic patients with I° and of uremic patients with II° hyperparathyroidism provides a better insight into the abnormal regulation of the endocrine function and growth of this tissue. The down-regulation of the CaR receptor could play an important role in the abnormal secretory and growth patterns of parathyroid glands in these pathologies. A better understanding of the underlying abnormalities should lead to a more appropriate clinical management.

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